SYNTHESIS OF ANTIOXIDANT PHENOLIC COMPOUNDS IN SEED SPROUT MODELS IN RESPONSE TO PLANT ELICITORS AND STRESSORS

A Dissertation

by

BOLIVAR ALEJANDRO CEVALLOS CASALS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Food Science and Technology

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Approved by:

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ABSTRACT

Synthesis of Antioxidant Phenolic Compounds in Seed Sprout Models
in Response to Plant Elicitors and Stressors. (August 2006)
Bolivar Alejandro Cevallos Casals, B.S., Texas A&M University;
M.S., Texas A&M University
Chair of Advisory Committee: Dr. Luis Cisneros-Zevallos

This study presents seeds as models for enhancing the levels of phytochemicals with health benefits. Increased understanding of the mechanisms of action of phytochemical synthesis would allow further seed manipulation for obtaining the desired quantity and quality of phytochemicals.

Dark germination, temperature, wounding, ultraviolet (UV) light and chemical elicitors (i.e. sucrose, chitosan, hydrogen peroxide) enhanced the levels of phenolic antioxidants in seedling models. All 13 seed species studied accumulated phenolic antioxidants during germination, with mungbean seed having the greatest accumulation.

Temperature treatments on mungbean seeds indicated a positive relation with phenolic accumulation in the temperature range of 10°C to 42°C, while wounding treatments showed that if applied right after imbibition, the faster the accumulation rate of soluble phenolics is. When wounding was applied at temperatures ≥ 25°C, there were enhancements on soluble phenolics at initial germination stages with decreases at later stages due to a possible lignification process.

Increases in coumestans and flavanones were observed in response to wounding and UV-C treatments; increases in flavonols were noted for UV-C stress only.

During mungbean germination there was increased phenolic synthesis of lignin precursors and phytoalexins. Results indicated that the phenolic synthesis was catalyzed by phenylalanine ammonia lyase (PAL) and mediated by reactive oxygen species (ROS) produced by membrane bound NADPH-oxidase.

Induction of phenolics in mungbean seeds due to UV-C stress showed a mechanism similar to that of dark germination; however, higher inductions of hydrogen peroxide, PAL, soluble phenolics, cell wall-bound phenolics, lignin, respiration rate and guaiacol peroxidase (POX) were observed.

From NADPH-oxidase inhibition studies we observed a reduction in the activity of POX, thus suggesting an activation/synthesis of POX due to ROS. This inhibition was lower for UV-C treated seeds, which indicates that induction of POX could be due to other signaling molecules in addition to those from ROS produced by NADPH-oxidase (e.g. ROS induced by water ionization due to UV-C).

In general, hydrogen peroxide is presumed to be the signal molecule mediating phenolic synthesis responses in germinating seeds, as well as playing a possible role in the mediation of seedling growth.

DEDICATION

To my beloved wife, Yvette, and my precious children, Raphaela and Matias. Every minute shared with you inspires me and gives me the energy to successfully overcome any obstacles and face new challenges.

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to my advisor and graduate committee chairman, Dr. Luis Cisneros-Zevallos, for his guidance, advice, motivation, support, and friendship. I would also like to extend my appreciation to Dr. Ronald Richter, Dr. David Byrne and Dr. Weston Porter for serving as committee members and helping me fulfill my doctoral studies.

Thanks to my lab-mates: Basilio, Emilio, Bernie, Lou, Evie, Carla, David, Giuliana, Marcia, Romina, Alex, Gaby, and Maria Rosa, for their friendship and help. Thanks to the Department of Horticultural Sciences and the Intercollegiate Faculty of Food Science and Technology for their support during all my academic studies at Texas A&M University.

I am deeply indebted to my parents, Mariangeles and Bolivar for their support and unconditional love at all times.

Many thanks to the donors of several scholarships I have received, and to the VFIC and the Tom Slick Fellowship Committee for their funding.

Special thanks to God for giving me the energy and faith to face all obstacles and challenges.

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CHAPTER I

INTRODUCTION

Phenolic compounds are secondary metabolites synthesized in plants for different growth and protection functions. They possess an aromatic ring bearing one or more hydroxyl substituents (Shahidi and Naczk 1995). Included in this category are phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Harborne 1993, Croteau and others 2001). Compounds such as lignins, lignans and suberins require phenolic precursors for their synthesis (Croteau and others 2001). Phenolics are important during normal plant growth and development as well as in response to biotic and abiotic aggressors. Some *in situ* properties conferred to phenolics include antimicrobial, UV protectant, antifeedant, pollinator attractant, allelopathy, and mechanical support (Dixon and Paiva 1995, Taiz and Zieger 1998a, Croteau and others 2001). Apart from their important roles in the plant, phenolic compounds have been widely studied and confirmed to possess diverse bioactive properties which could be beneficial to human health. These compounds have been related to reduction in the risks of cancer, heart disease, and diabetes; inhibition of plasma platelet aggregation, cyclooxygenase (COX) activity, and histamine release; as well as to in vitro antibacterial, antiviral, anti-inflammatory, and antiallergenic activities (Yang and others 2001; Shetty 2004; Yao and others 2004; Oak and others 2005). The benefits towards many of these conditions come in part through the antioxidant characteristic of phenolic compounds; therefore, the importance of their quantification, identification and evaluation as antioxidants.

Due to the potential significance of phenolic antioxidants for the prevention of a wide range of degenerative physiological processes, the concept of increasing their natural levels by the use of controlled postharvest abiotic stresses and elicitors in fruits and vegetables was recently proposed as an alternative to genetic modifications (Cisneros-Zevallos 2003). Specific attempts have been made through the use of

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modified atmospheres (Zheng and others 2003), ultraviolet (UV) light (Cantos and others 2003), wounding (Reyes and Cisneros-Zevallos 2003), plant hormones (Heredia and others 2001) and microbial elicitors (McCue and Shetty 2002a). Most of these treatments have elicited a response of increased phenolic synthesis; however the mechanism of action, including the identity of the signaling molecules, is not well understood. Identifying the responsible signaling molecules would allow better control of phenolic synthesis.

Many compounds such as phytohormones (ethylene, jasmonic acid [JA], abscisic acid), mitogen-activated protein kinases (MAPK), reactive oxygen species (ROS), salicylic acid, systemin, oligosaccharides, as well as electrical pulses and hydraulic waves have been proposed to play a role in wound signaling during wounding stress (Saltveit 2000, Leon and others 2001, Rakwal and Agrawal 2003). Among these proposed signaling mechanisms, the main ones seem to be ethylene, JA, and ROS production (Saltveit 2000, Leon and others 2001, Zhao and others 2005).

Regarding UV radiation, there seems to be expression of several plant defensive genes that are normally activated after wounding (Conconi and others 1996, Stratmann 2003). Similar to wounding, production of JA, ethylene, and ROS have been observed during UV radiation (Conconi and others 1996, Hollosy 2002, Rakwal and Agrawal 2003, Stratmann 2003). The precise mechanism of action of phenylpropanoid induction in response to UV, especially UV-C (200-280 nm), still awaits elucidation.

In this doctoral research we present the use of seedlings as models for potential phenolic and antioxidant activity enhancement by the use of controlled abiotic stresses and elicitors. Seeds serve as appropriate models for understanding elicitor and stress effects on final phenolic synthesis due to their high metabolic state during germination (Shetty 2004, Chalker-Scott 1999). From the moment the seed breaks dormancy, the growing seedling is exposed to harmful environmental conditions, thus signaling protective responses through the synthesis of phenolics and other compounds (Taiz and Zieger 1998a). The addition of controlled elicitors and stresses would further enhance this protective response.

Continuous measurements of antioxidant activity throughout the study helps determine changes in bioactivity during germination and in response to chemical elicitors, wounding and two types of UV light (UV-B: 280-320 nm and UV-C 200-280 nm).

The specific objectives were to study the synthesis of phenolic antioxidants during modified germination conditions and upon exposure to elicitors and stressors known to enhance phenolic synthesis. In addition, the potential secondary signaling molecules responsible for phenolic synthesis under wounding and UV-C light treatments were studied. The study is presented in four main chapters.

Chapter II evaluated the range of phenolic synthesis responses during germination of 13 seeds from different species. Their phenolic synthesis responses were also evaluated when subjected to different chemical elicitors and two types of UV light (UV-B and UV-C). From this work, mungbean was selected as appropriate seed model for subsequent research.

In Chapter III, phenolic synthesis in selected mungbean seeds was studied in response to different growing temperatures, wounding treatments and a combination of temperature and wounding.

Chapter IV gives an indication of the type of phenolics synthesized during mungbean germination and after UV-C and wounding stresses, and explores potential signal molecules mediating these responses. Selected signaling candidates were targeted in Chapter V.

In Chapter V, the mechanisms of action for phenolic synthesis in mungbean due to dark germination and UV-C are determined. A detailed study of signal transduction towards phenolic synthesis during germination and after UV-C stress is presented for understanding the sequence of events and elucidating the mechanisms of action. After evaluating the signal transduction events, select targets were inhibited for determining their contribution to the phenolic synthesis response. Enzyme inhibitor studies helped elucidate the possible mechanism by which mungbean seeds synthesize phenolic compounds during germination and in response to UV-C stress.

This dissertation should provide a thorough understanding on the mechanism of action of phenolic synthesis during different natural and man-made conditions and their specific roles for plant protection and growth. Due to this increasing understanding of plant response mechanisms, we intend to take advantage of plants as phenolic-producing factories and exploit them as sources of nutraceutical compounds.

CHAPTER II

GERMINATION AND EXPOSURE TO UV LIGHT AND CHEMICAL ELICITORS ENHANCE THE PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF SEEDS OF 13 PLANT SPECIES

Synopsis

There was increased phenolic antioxidant synthesis as germination progressed in the seeds of 13 plant species studied. Increases in phenolics and total antiradical capacity (TAC) on a dry weight basis (DB) from dormant to imbibed seed were on average ~60% and ~166%, respectively. From the total antioxidant activity accumulated within 7 days of germination, a ~48% was accumulated during water imbibition for 17 h. The increases in phenolic content on a DB from dormant seed to 7 d sprout were in the order mungbean (2010%) > fava (586%) > wheat (535%) > fenugreek (530%) > mustard (435%) > alfalfa (409%) > sunflower (271%) > soybean (201%) > broccoli (186%) > lentil (185%) > radish (63%) > kale (-11%). For TAC on a DB, the increases were in the order mungbean (1928%) > alfalfa (943%) > fava (919%) > fenugreek (681%) > mustard (566%) > wheat (433%) > broccoli (290%) > sunflower (261%) > soybean (175%) > radish (117%) > lentil (17%) > kale (16%). Imbibed seeds had higher phenolic specific TAC than dormant seeds and 7 d sprouts, indicating the presence of phenolics with higher reactivity towards DPPH radicals. Seven day sunflower sprouts had higher TAC on a DB (40202 ug Trolox equivalents g^{-1}) than the other seeds (1456 to 25991) and than a blueberry reference (35232) as well as higher reaction efficiency of phenolics against DPPH radicals. Phenolic contents of 7 d sprouts on a wet weight basis (WB), DB, and per seed basis (PSB) ranged from 122 (alfalfa) to 555 mg chlorogenic acid equivalents (CAE) $100g^{-1}$ (sunflower), from 490 (lentil) to 5676 mg CAE $100g^{-1}$ (mustard), and from 0.02 (alfalfa) to 6.4 mg CAE $seed^{-1}$ (fava bean), respectively. Chemical elicitation responses were in the order sucrose > chitosan ≈ gellan gum > proline. With sucrose elicitation, fava bean hypocotyl had 41%, 47%, and 76% higher

phenolic content PSB than controls, after 2, 5 and 8 days, respectively. For UV-B and UV-C treatments, radish and mungbean 7 d sprouts had 13% and 66% higher TAC on a WB than controls, respectively. A dose-response effect of UV-C on phenolic synthesis of mungbean seeds was observed.

Introduction

Germination starts when the dry seed begins to take up water and is completed when the embryonic axis elongates. At this point reserves within the storage tissues of the seed are mobilized to support seedling growth. Within minutes of water imbibition, metabolic activity is resumed, including mitochondrial respiration and protein synthesis, initially using structural and enzymatic components synthesized during development and conserved in the dry state. Later during imbibition, extant RNAs are replaced by de novo transcription and turnover. New mRNAs are produced, as well as transcripts encoding proteins to support basic metabolism (Bewley and others 2001).

From the moment the seed breaks dormancy, the growing seedling may be exposed to harmful environmental conditions, which signal protective responses through the synthesis of phenolics and other compounds (Taiz and Zeiger 1998a). This protective response may further be enhanced by the addition of controlled elicitors. Among these elicitors, UV light and chemical elicitors, especially carbohydrate-based elicitors, have shown significant enhancement of phenolic synthesis in different plant tissues, including seedlings (Cantos and others 2003, McCue and Shetty 2002a). UV light has shown to increase the activity of enzymes from the pentose phosphate pathway (PPP) (Shetty and others 2002), shikimic acid pathway (McCue and Conn 1990; Sharma and others 1999) and phenylpropanoid metabolism (Gitz III and others 1998; Chalker-Scott 1999; Frohnmeyer and Staiger 2003), important pathways for phenolic synthesis. Chemical elicitors such as chitosan and gellan gum have shown to induce phenolic synthesis and lignification, possibly through activation of the genes for plant defense reactions against microbial pathogens (Reddy and others 1999; McCue and Shetty 2002a), and proline, through overexpression of PPP (Shetty and others 2003). Other

carbohydrate sources could enhance phenolic synthesis by increasing the overall metabolic flux. For example, sucrose is the major translocated sugar in plants, is the carbon form that most non-photosynthetic tissues import, and is thought to be the true sugar substrate for glycolysis (plant respiration) (Taiz and Zeiger 1998b); therefore having extra sugar substrates for metabolic reactions could increase phenylpropanoid metabolism.

It is clear that one of the responses of growing seedlings to unfamiliar natural and controlled conditions is to synthesize phenolics, among other compounds; however, it is not clear how the level of phenolics, especially phenolic antioxidants, vary throughout seed germination as well as upon exposure to controlled physical and chemical elicitors. We hypothesized that phenolic synthesis will change with germination stage and with elicitor type and that antioxidant activity of the synthesized phenolics will change throughout germination and in response to chemical elicitors and UV light. Changes in phenolic synthesis and antioxidant activity would indicate seed preparation towards adverse conditions. Identifying germination stage or elicitor treatment where the level of phenolic antioxidants is optimized would be attractive for the growth of edible sprouts with enhanced nutraceutical properties. In the present study, our approach was to test 13 different seeds for the levels of phenolic compounds and their antioxidant properties at different germination stages as well as in the presence of chemical elicitors (i.e. chitosan, gellan gum, proline and sucrose) and UV light (i.e. UV-B: 280-320 nm, UV-C: 200-280 nm).

Materials and Methods

Materials

Fava bean (*Vicia faba*), sunflower (*Helianthus annuus*), green lentil (*Lens esculenta*), onion (*Allium cepa*), mung bean (*Vigna radiata* L. Wilczek), mustard (*Brassica juncea*), radish (*Raphanus sativus* 'Daikon'), wheat (*Triticum aestivum*), alfalfa (*Medicago sativa*), kale (*Brassica napus pabularia* 'Red Russian'), fenugreek (*Trigonella foenum-graecum*) and soybean (*Glycine max* 'Butterbeans') seeds were

purchased from Johnny's Selected Seeds (Winslow, ME, USA), while broccoli (*Brassica oleracea* var. Italica 'Decicco') and cabbage (*Brassica oleracea* var. Capitata 'Red Acre') seeds from Holmes Seeds (Canton, OH).

Chlorogenic acid, trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Seed germination

Seeds were sterilized with 70% ethanol for 2.5 min, followed by 2.5% sodium hypochlorite for 15 min (Huang and others 2003). Ethanol and sodium hypochlorite were removed with four rinses of sterile water. After disinfection, seeds were allowed to imbibe water at 18 °C for 17 h. Then water was removed and seeds were dark-germinated in sterile petri plates with humidified Whatman No.2 filter papers at 18 °C. Filter paper was kept moist by spraying with sterile water as needed.

Seeds were assayed for dry matter, total phenolics and total antiradical capacity (TAC) through time. For some tests fava bean was further separated into hypocotyl and cotyledon sections. Cotyledon section included the seed coat, while hypocotyl section included hypocotyl and epicotyl tissues (Figure 1). Depending on their size and weight, 3 (fava), 8 (soybean), 10 (mungbean, sunflower), 15 (lentil), 20 (fenugreek), 25 (radish), 30 (kale, wheat, cabbage), 40 (broccoli, onion), or 70 (alfalfa, mustard) seeds represented one replicate. Three to six replicates were conducted for each assay.

UV and chemical treatments

For UV treatments, seed sprouts were exposed to UV-B (90W for 2 h) or UV-C (120W for 35 min) light 3 days after water imbibition. For chemical treatments gellan gum (0.1%), sucrose (0.2% or 0.3%), chitosan (0.6 %) and proline (0.003%) were dissolved in water and used as the imbibition solution. In addition, dose-response studies were conducted on mungbean seeds with sucrose (0.1% to 10%), chitosan

(0.001% to 1%) and UV-C (180W for 10 to 40 min, 20 °C). After UV and chemical treatments, samples were dark-germinated in sterile petri plates for up to 8 days.

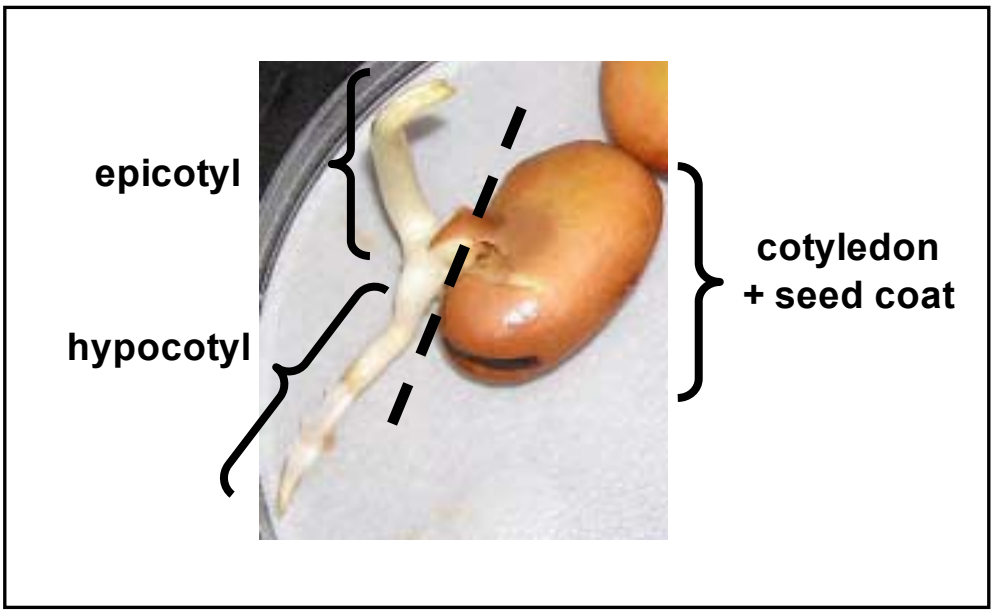

Figure 1 – 8 d old germinated fava bean showing its different section components. Dashed line indicates were the cut was made for sampling of hypocotyl section. Hypocotyl section included the hypocotyl and epicotyl, while cotyledon section included the cotyledon and seed coat.

Total soluble phenolics

Total soluble phenolic content of methanolic extracts was assayed as described by Cevallos-Casals and Cisneros-Zevallos (2003) using Folin-Ciocalteu reagent with final reaction measurements conducted at 725 nm. Total phenolics were expressed as mg chlorogenic acid equivalents (CAE) 100 g^{-1} wet basis (WB), dry basis (DB) or per seed basis (PSB), based on a standard curve.

Total antiradical capacity (TAC)

TAC of phenolic compounds was adapted from Brand-Williams, Cuvelier, and Berset (1995). The same methanol extract as for phenolics was used. A total of 150 ul of sample (equivalent methanol volume to control) reacted with 2850 ul DPPH (98.9 uM in methanol) in a shaker covered with aluminum foil at 20 °C. Readings at 515 nm were taken after 20 h reaction time. The change in absorbance was used and results were expressed as ug Trolox equivalents g^{-1} WB, DB or PSB, from a standard curve. In addition, specific antioxidant capacity (specific TAC) was defined as the ratio of total antiradical capacity/total soluble phenolics and expressed as μg Trolox equivalents mg^{-1} CAE. The specific antioxidant capacity provides information on the effectiveness of phenolics to neutralize free radicals. A higher specific TAC means phenolic compounds have a higher capacity to stabilize free radicals.

Isolation of phenolic compounds with C-18 resin

For confirming that phenolic compounds in methanol extracts were the major compounds reacting with DPPH and Folin-Ciocalteu reagents, phenolic compounds from representative seeds were isolated with C-18 cartridges and reacted with DPPH and Folin-Ciocalteu. Methanol extracts were concentrated to dryness on a Speed Vac Concentrator (Model SV0-100H, Savant Instruments, Inc., Hicksville, NY) at 35 °C attached to an aspirator pump. Samples were re-diluted with acidified (0.01% HCl) water. Aqueous samples were applied to Sep-Pak Plus C18 cartridges (Waters Assoc., Milford, MA), previously activated with acidified methanol followed by acidified water.

Water-soluble compounds, including sugars and acids, were eluted with acidified water and phenolics were recovered with acidified methanol.

Analysis of variance and covariance

One-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Means were compared with Duncan's Multiple Range Test and LSD test at $\alpha = 0.01$ and 0.05.

Results and Discussion

Changes in total phenolics and antioxidant capacity of seeds at different germination stages

The values of total phenolics and TAC for a dry or dormant seed would indicate the amount of phenolic antioxidants synthesized while the seed was attached to the parent plant, while for imbibed seed and 7 d sprout the total phenolics and TAC values indicate synthesis of phenolic antioxidants after dormancy (Figures 2, 3).

Phenolic contents and their antioxidant activity during germination were expressed on a wet weight basis (WB), dry weight basis (DB) or per seed basis (PSB). Concentrations expressed on a WB may be influenced by changes in moisture content presenting a dilution effect on the synthesis of phenolics. When results are expressed on a DB, the moisture component is eliminated, and on PSB a potential understanding of total yields per seed unit is obtained; therefore our preference for presenting results in DB and/or PSB.

Dormant stage (dry seed)

Phenolic content and TAC values on a DB for dormant seeds represented an average contribution of ~32% and ~31%, respectively, compared to sprouts after a 7 d germination process, indicating that most of the synthesis of phenolic antioxidants occurs during imbibition and seed growth (Figures 2B, 3B, 4). Phenolic content on a WB for dormant seeds was higher than that for imbibed seeds and 7 d sprouts (except for fava and mungbean), suggesting a dilution effect of phenolics after water imbibition and growth due to an increase in water absorption (Figure 2C, Table 1). On the other hand,

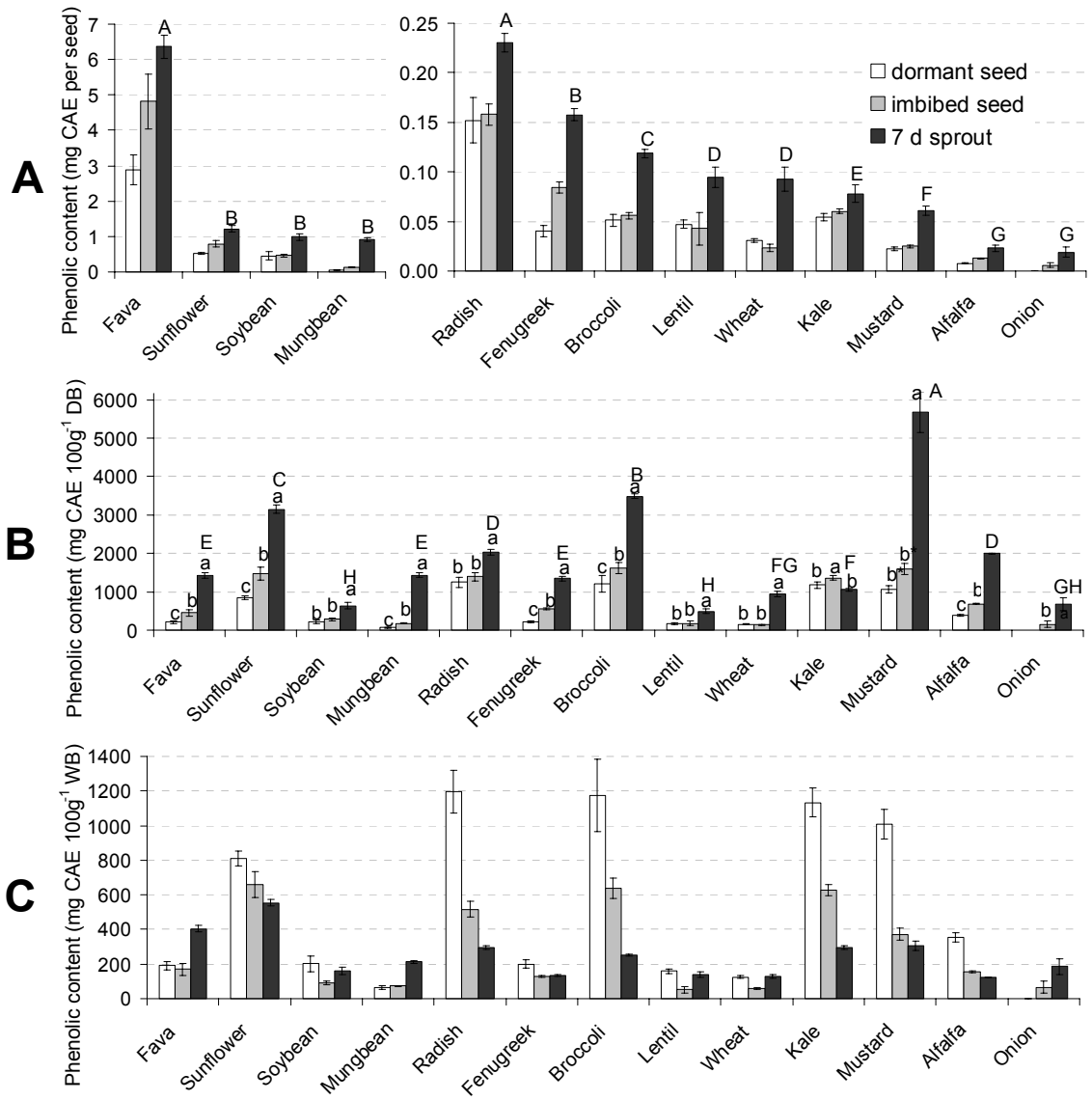

Figure 2 – Phenolic content of seeds of 13 plant species at three different germination stages grown at 18 °C. A = per seed basis (PSB), B = dry basis (DB), C = wet basis (WB). Phenolic content was expressed in mg chlorogenic acid equivalents. 7 d sprouts with similar upper case letters within each figure are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Seed stages with the same lower case letter are not significantly different ($\alpha = 0.05$ with Duncan test) from each other within the same plant species. *Dormant and imbibed mustard seed were significantly different ($\alpha = 0.05$ with Duncan test) when ran indepently of 7 d sprout. Total phenolic values for dry onion seeds could not be obtained. Bars show the average of 3 to 6 replicates ± standard deviation.

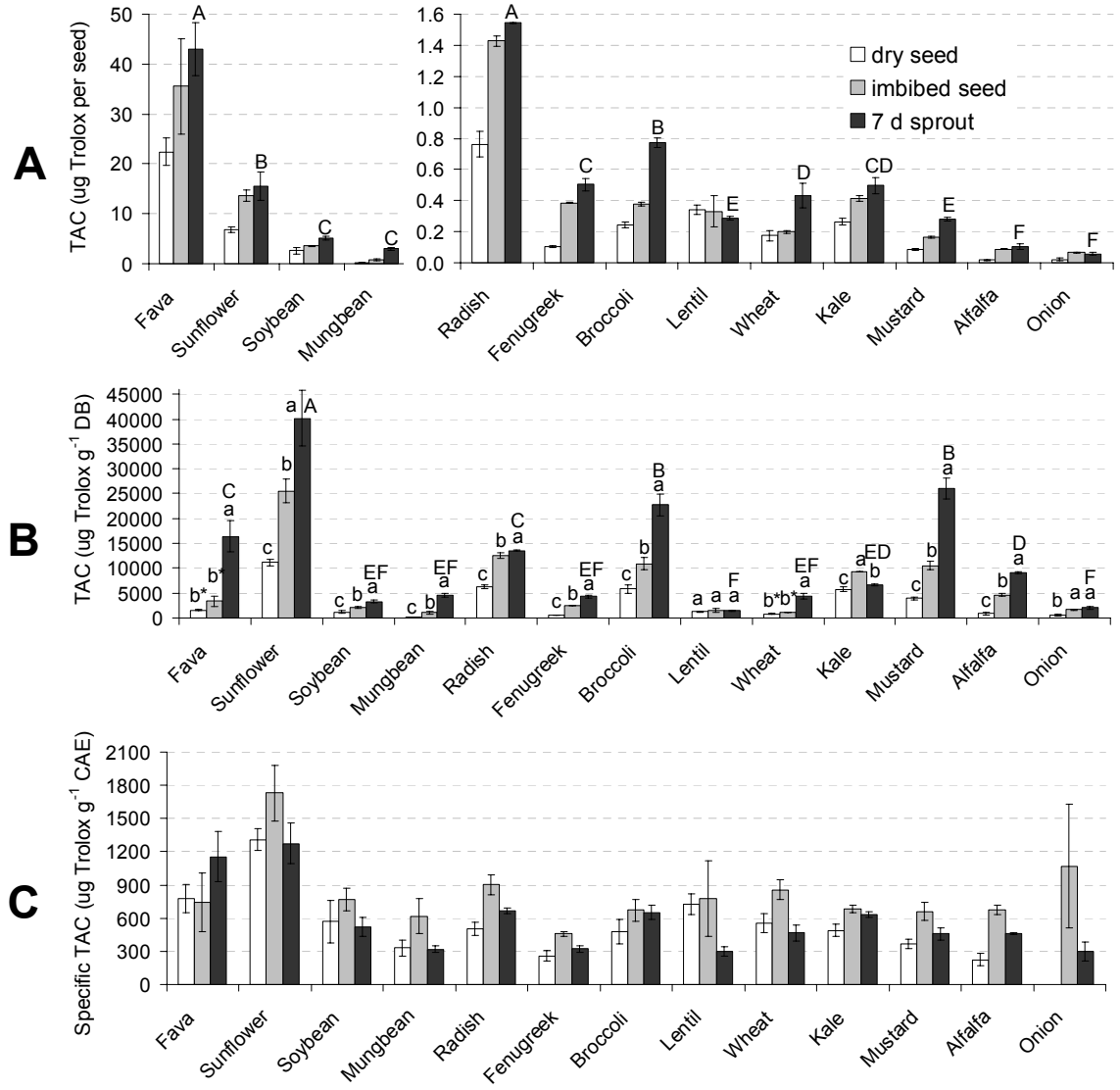

Figure 3 – TAC of seeds of 13 plant species at three different germination stages grown at 18 °C. A = per seed basis (PSB), B = dry basis (DB), C = TAC based on phenolic basis. TAC was expressed in ug Trolox equivalents. 7 d sprouts with similar upper case letters within each figure are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Seed stages with the same lower case letter are not significantly different ($\alpha = 0.05$ with Duncan test) from each other within the same seed. *Dormant and imbibed stages for fava and wheat seeds were significantly different ($\alpha = 0.05$ with Duncan test) when ran indepently of 7 d sprout stage. Specific TAC values for dry onion seeds could not be obtained. Bars show the average of 3 to 6 replicates ± standard deviation.

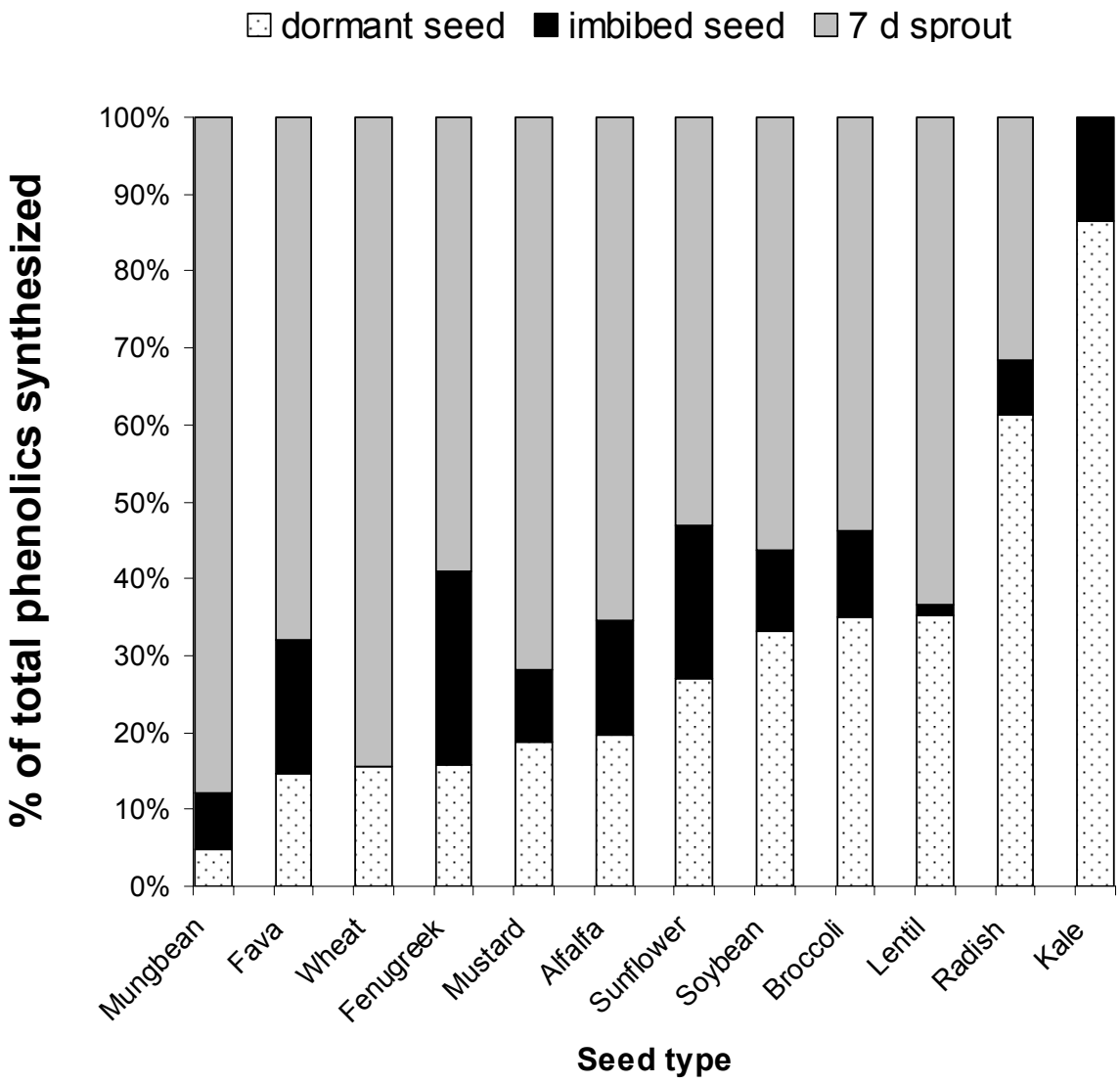

Figure 4 – Percentage of phenolics synthesized at the different germination stages: dormant stage, imbibition stage and 7 d sprout stage.

Table 1 – Changes in seed weight and moisture throughout germination at 18 °C.

Seed	Weight (mg)			% moisture		
	dormant seed	imbibed seed	7 d sprout	dormant seed	imbibed seed	7 d sprout
Fava	1503 ± 21	2860 ± 205	3452 ± 946	7.4	62.7	71.5
Sunflower	64 ± 5	120 ± 1	307 ± 1	4.6	55.4	82.4
Soybean	222 ± 7	499 ± 31	622 ± 101	4.8	66.7	74.9
Mungbean	79 ± 3	176 ± 4	447 ± 3	6.4	58.5	85.2
Radish	13 ± 0.6	31 ± 1	83 ± 7	3.8	62.8	85.4
Fenugreek	20 ± 1	66 ± 2	153 ± 2	5.8	76.5	90.1
Broccoli	4.4 ± 0.3	8.8 ± 1	45 ± 1	3.7	60.3	92.8
Lentil	29 ± 1	81 ± 12	130 ± 5	7.3	70.7	71.9
Wheat	25 ± 2	40 ± 3	99 ± 9	15.4	57.0	86.3
Kale	4.8 ± 0.0	9.6 ± 0.4	11 ± 1	3.7	53.9	72.1
Mustard	2.2 ± 0.0	6.8 ± 1	19 ± 2	4.9	76.7	94.6
Alfalfa	2.1 ± 0.0	8.3 ± 0.3	19 ± 1	10.0	77.5	93.9
Onion	4.1 ± 0.1	9.4 ± 1	15 ± 0.1	5.7	57.1	72.6

Values for weight show the average of 3 to 6 replicates ± standard deviation.

phenolic content and TAC on a DB and PSB showed a consistent trend, where 7 d sprouts > imbibed seeds > dormant seeds, indicating a continued phenolic synthesis throughout the germination process which could serve as protection against environmental factors and for structure-giving (Figures 2A, 2B, 3A, 3B).

Water imbibition stage

Imbibition stage showed to be a highly active period of phenolic antioxidant synthesis. Increases in phenolics and TAC on a DB from dormant to imbibed seed were in average ~60% and ~166%, respectively (Figures 2B, 3B). Of the total phenolics and TAC accumulated within 7 days of germination, synthesis during seed imbibition (17 h) accounted for ~22% and ~48%, respectively (Figures 2B, 3B), showing a high rate of phenolic antioxidant synthesis. In addition, the average increases in TAC on a DB and PSB from dormant to imbibed seeds were higher than from imbibed seeds to 7 d sprouts (except for broccoli and wheat) (Figures 2A, 2B, 3A, 3B). Average DB contents of phenolics and TAC for imbibed seeds were ~41% and ~59%, respectively, of the total contents of 7 d sprouts (Figures 2B, 3B). Wheat was the only seed not experiencing an increase in total phenolics during imbibition.

The specific TAC (normalized to phenolic content) was usually higher for imbibed seeds as compared to dormant seeds, with an increase up to two-fold in the case of alfalfa (Figure 3C). This indicates that phenolic compounds with a higher number of DPPH reactive hydroxyl groups were been synthesized during water imbibition. This was further confirmed by plotting phenolic content against TAC for all seeds, except sunflower (Figure 5). Results show that the slope of the linear regression fit for imbibed seeds was statistically higher ($\alpha = 0.01$ ANCOVA) than that for dormant seeds, indicating that at similar phenolic contents, TAC will be higher for imbibed seeds. A higher specific TAC for imbibed seeds could suggest that the first steps the seed response machinery takes after breaking dormancy is to synthesize phenolic compounds with higher than normal antioxidant activity so as to protect hypocotyl growth against

oxidative reactions and compounds generated and signaled by environmental factors such as UV light, ozone, plant pathogens, and other harmful conditions.

Sprout growth stage (7 d sprout)

Approximately 59% of the total phenolics found in 7 d sprouts were synthesized after imbibition and these synthesized phenolics accounted for ~41% of the final TAC (Figures 2B, 3B, 4). The increases in phenolic content on a DB from dormant seed to 7 d sprout were in the order mungbean (2010%) > fava (586%) > wheat (535%) > fenugreek (530%) > mustard (435%) > alfalfa (409%) > sunflower (271%) > soybean (201%) > broccoli (186%) > lentil (185%) > radish (63%) > kale (-11%) (Figure 2B). These results show mungbean to be the seed with the greatest increase in phenolics since ~95% of the total phenolics were synthesized during imbibition and 7 d growth (Figure 4). On the other hand, kale was the only seed showing a decrease in phenolic concentration on a DB from imbibition stage to 7 d sprout (Figure 2B), which could be explained by absence of phenolic synthesis, utilization of phenolic compounds for protection functions, exudation of phenolic compounds, or a combination of these. For TAC on a DB, the increases were in the order mungbean (1928%) > alfalfa (943%) > fava (919%) > fenugreek (681%) > mustard (566%) > wheat (433%) > broccoli (290%) > sunflower (261%) > soybean (175%) > radish (117%) > lentil (17%) > kale (16%) (Figure 3B). These increases in phenolic content and TAC show possible important roles of phenolics during seed germination, as well as the potential enhancement of the nutraceutical value of seeds by germination.

After 7 day growth, phenolics on average, had a lower reaction efficiency against DPPH radicals [$y = 5.267x$, $R^2 = 0.807$ (data not shown)], as compared to imbibed seeds [$y = 7.1249x$, $R^2 = 0.9492$ (Figure 5)]. This could be caused by the oxidation of antioxidant phenolics and their utilization as precursors of lignin or lignan structures (Ascensao and Dubery 2003). It is also possible that the synthesis rate of phenolics with high antioxidant activity decreased or stopped and the synthesis rate of phenolics with lower number of DPPH reactive OH groups, increased.

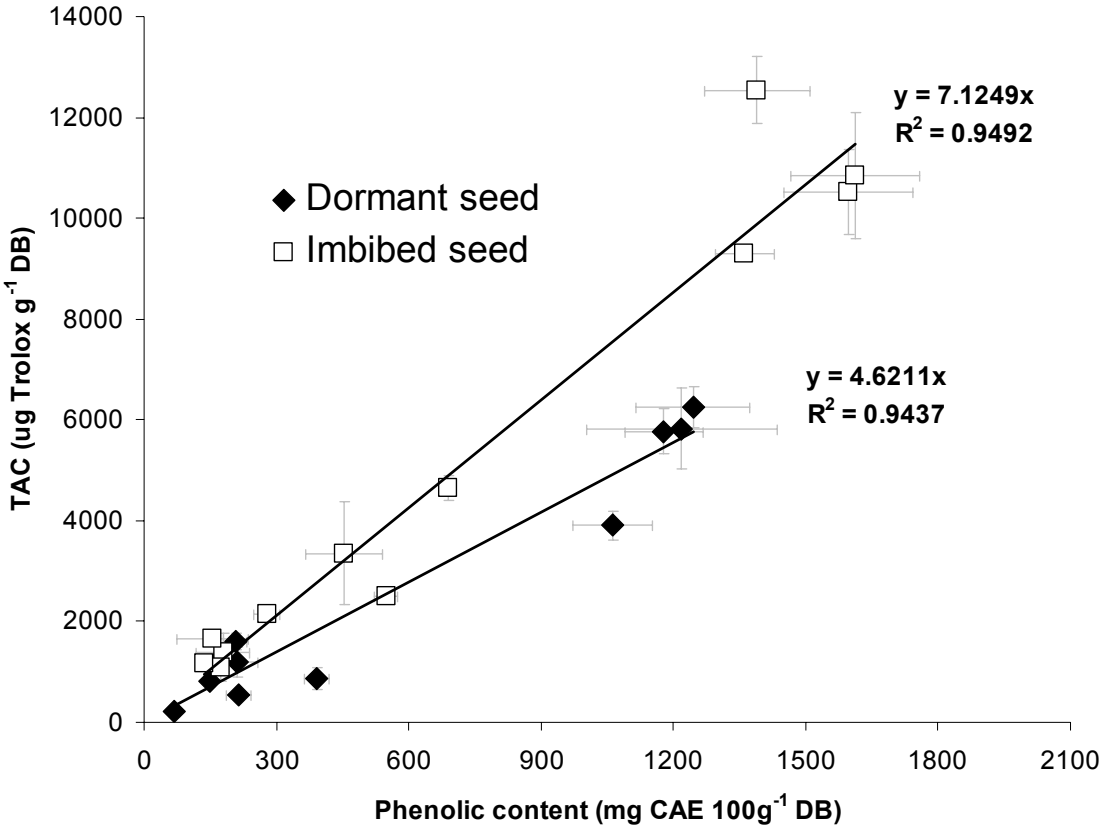

Figure 5 – Increase in antiradical efficiency during seed imbibition. Lines show linear regression fittings of the data. Data shows all seeds except sunflower. Both curves significantly different at α=0.01 using ANCOVA (testing for slopes). Values show the average of 3 to 6 replicates ± standard deviation.

When the phenolic compounds of sunflower, fava, radish and mungbean at dormant, imbibed and 7 d sprout stage were isolated using C-18 cartridges, no significant difference (p-value > 0.05) was found between the phenolic specific TAC of C-18-purified phenolic compounds as compared to the phenolic specific TAC of methanolic extracts. These results confirm that phenolic compounds are responsible for most of the antioxidant properties of the sample methanolic extracts, thus reinforcing the validity of expressing TAC on a phenolic basis.

Differences in phenolic content and TAC between the seeds of 13 plant species at different germination stages

Sunflower seeds showed high phenolic content and the highest TAC on a WB, DB and phenolic basis (PB) of all seeds at the evaluated stages (Figures 2, 3). Imbibed sunflower seeds showed higher TAC on a DB (25566 ug Trolox g^{-1}, Figure 3B) and phenolic specific TAC (1731 ug Trolox g^{-1}, Figure 3C) than the rest of the imbibed seeds and higher than previously published values, including red sweetpotato, purple corn and blueberry (Cevallos-Casals and Cisneros-Zevallos 2003). Seven day sunflower sprouts showed significantly higher TAC on a DB (40202 ug Trolox g^{-1}) than the other seeds (1456 to 25991 ug Trolox g^{-1}) and than that of a blueberry reference (35232 ug Trolox g^{-1}), considered to have the highest antioxidant activity of all fruits and vegetables tested (Cevallos-Casals and Cisneros-Zevallos 2003). Due to their high phenolic specific TAC values, sunflower seeds seem to possess phenolic compounds with molecular structures bearing a high number of DPPH reactive hydroxyl (OH) groups. The major phenolic compounds identified in sunflower seeds have been chlorogenic acid (55%), 1,4-di-O-caffeoylquinic acid or 1,5-di-O-caffeoylquinic acid (chlorogenic acid derivative) (30%), caffeoyl-pentahydroxycinnamoyl-quinic acid (chlorogenic acid derivative) (10%) and caffeic acid (4%) (Pedrosa and others 2000). Chlorogenic acid has been determined to have 6 reactive OH groups, a reactivity level higher than most phenolic compounds (Rice-Evans and Miller 1996, Rice-Evans, Miller and Paganga 1996). It is possible that the functional groups of the two chlorogenic acid derivatives further enhance the

molecular reactivity towards free radicals. According to Rice-Evans, Miller and Paganga (1996), at least two or three neighboring phenolic OH groups and a carbonyl group in the form of an aromatic ester, o-lactone, or a chalcone, flavanone or flavone are essential for achieving a high level of antioxidant activity.

Other seeds showing high phenolic content and TAC included radish, broccoli, mustard and fava (Figures 2, 3). Radish and broccoli had the highest phenolic concentration on a WB (Figure 2C) and on a DB (Figure 2B) among dormant seeds. Broccoli and mustard had the highest phenolic concentration on a DB (Figure 2B) among imbibed seeds, while mustard and broccoli had the highest phenolic concentration on a DB (Figure 2B) among 7 d sprouts. The amount of phenolics and TAC on a PSB at all germination stages was highest for fava bean, due to its large size (~1.5g/seed) (Figures 2A, 3A). Even though sunflower was ranked fourth in total weight per seed, it was ranked second, following fava, in amount of phenolics and TAC contributed per seed at the different stages tested (Table 1; Figures 2A, 3A).

Phenolic contents of 7 d sprouts on a WB, DB, and PSB ranged from 122 (alfalfa) to 555 mg CAE $100g^{-1}$ (sunflower), from 490 (lentil) to 5676 mg CAE $100g^{-1}$ (mustard), and from 0.02 (alfalfa) to 6.4 mg CAE $seed^{-1}$ (Fava bean), respectively. For comparison purposes, blueberries, known to have high phenolic content and antioxidant activity have phenolic concentrations ranging from 292 to 672 mg CAE $100g^{-1}$ on a WB and from 1956 to 4202 mg CAE $100g^{-1}$ on a DB (Cevallos-Casals and Cisneros-Zevallos 2003). The high level of phenolic antioxidants in most of the sprouts tested suggests that consumption of fresh sprouts could potentially provide similar antioxidant benefits to those of fresh blueberries.

The effect of chemical elicitors on phenolic synthesis and seed growth of wheat and fava bean through germination

During tests with chemical elicitors, fava bean was separated into cotyledon and hypocotyl for determining effects on these different seed parts with different functions and metabolic activities (Figure 6). Analyzing separate plant tissues is important for

studying the distribution of active compounds and for maximizing the use of their beneficial physiological functions (Yoshimoto and others 1999). This information can also facilitate the design of appropriate extraction processes or improve existing ones (Cevallos-Casals and Cisneros-Zevallos 2003).

Phenolic and seed weight changes through time

Day 0 data was not included in Figure 6 since it was not possible to generate values for dry and imbibed fava bean separated into cotyledon and hypocotyl due to the start of hypocotyl growth one day after imbibition. Figures 6B and 6C show that phenolic content PSB and seed weight consistently increased through time for all treatments applied on wheat and fava bean. Regarding phenolic content on a WB for fava bean cotyledon and for whole wheat, there was an increase from day 2 to day 5, with a decrease from day 5 to day 8 (Figure 6A). Fava bean hypocotyl experienced an increase from day 5 to day 8, but at a lower rate than the increase from day 2 to day 5. The same occurred when results were expressed on PSB, a faster accumulation rate of phenolics from day 2 to day 5 than the accumulation from day 5 to day 8 (Figure 6B). Compared to the cotyledon, the hypocotyl has a higher metabolic activity, which could explain why phenolic synthesis occurred at higher rates than water absorption and synthesis of other compounds at the assayed germination stages. As was observed with whole seeds (Figure 2C, Table 1) and less metabolically active tissues (cotyledon, Figure 6A), synthesis of phenolic compounds could be masked by buildup of other compounds (i.e. water) with faster accumulation rates that create a dilution effect, therefore the importance of presenting results in DB or PSB.

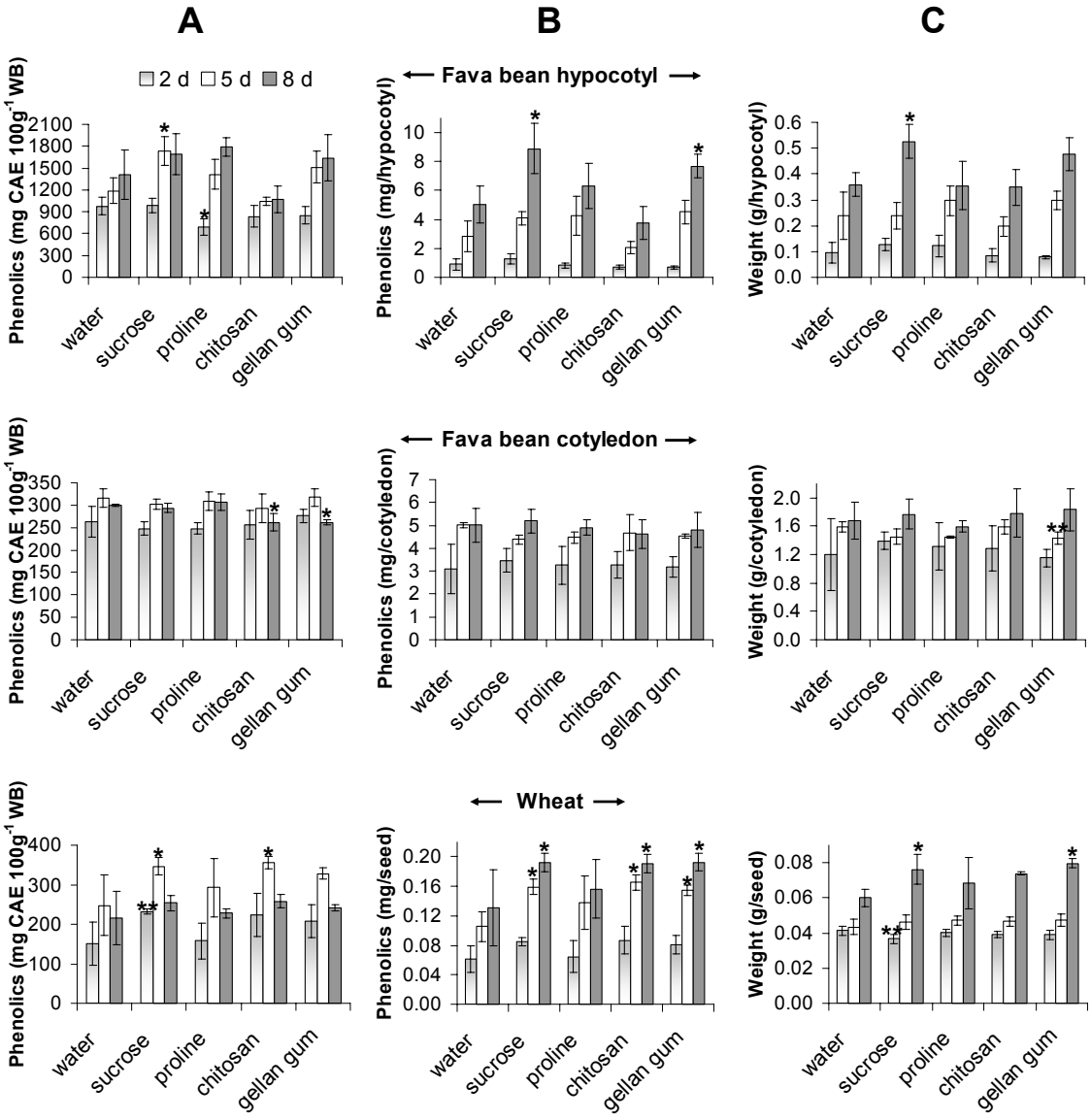

Figure 6 – Changes in phenolic content and seed weight of fava bean and wheat throughout germination in response to different chemical elicitors. A = wet basis (WB), B = per seed or per seed section basis (PSB), C = fresh weight per seed or seed section. Phenolic content was expressed in mg chlorogenic acid equivalents. *Significantly different from water control, α=0.05 with Duncan test; ** α=0.05 with LSD test. Elicitors were dissolved in the imbibition solution.

Overall, these results reinforce the importance of phenolic synthesis throughout germination for protecting the emerging hypocotyl against oxidative and other harmful conditions, including their potential role as cell wall strengtheners (Ascensao and Dubery 2003). We hypothesize that phenolic synthesis rates, especially synthesis of phenolic antioxidants, are higher at early germination stages for protecting a fragile growing hypocotyl and that at later germination stages net phenolic synthesis rates decrease due to lignin formation, a protective barrier against detrimental environmental conditions and a necessary compound for physical structure (Ascensao and Dubery 2003). With lignin protecting internal cell components the need for phenolic synthesis as protectors could be diminished. In addition, phenolic compounds could be increasingly esterified into the cell wall and later transformed into lignin, therefore not being extracted in methanol and quantified with the Folin-Ciocalteu assay. This could be verified in future studies by measuring individual soluble phenolics, cell wall bound phenolics and lignin. So far, previous studies have determined that lignin and enzymes related to lignin synthesis increase with seedling growth (Tuyet Lam and others 1996, Randhir and Shetty 2004).

Phenolic content and weight changes due to chemical elicitors

When exposed to different elicitors, the changes in phenolic content on fava bean cotyledon were much lower than that of the hypocotyl (Figure 6), reinforcing the higher metabolic activity of the hypocotyl. It has been proposed that when germination begins, metabolites are mobilized from the cotyledon to provide key precursors for the growing hypocotyl (Shetty and others 2001).

From the tested chemical elicitors, sucrose was the treatment that elicited the highest phenolic accumulation per seed. Sucrose-elicited fava bean hypocotyl had 41%, 44%, and 76% higher phenolic content PSB than water controls, after 2, 5 and 8 days, respectively, while sucrose on wheat generated 39%, 52% and 47% higher phenolic content PSB, at the same time intervals (Figure 6B). Sucrose is the major translocated sugar in plants and is the carbon form that most non-photosynthetic tissues import,

therefore these responses could indicate that sucrose enhanced the overall carbon metabolic flux and rate in these seeds thus favoring phenolic synthesis among other reactions.

In addition to sucrose, chitosan and gellan gum also elicited phenolic synthesis responses. On wheat sprouts, chitosan and gellan gum treatments yielded 45% to 57% higher phenolic contents PSB than water controls on 5 and 8 d sprouts, while on 8 d fava bean hypocotyl, gellan gum generated a 52% higher phenolic content PSB than water control (Figure 6B). Proline treatments did not show any significant increases in phenolic content. Mechanisms of the chitosan and gellan gum effects could be through elicitation of pathogenesis-related proteins (Reddy and others 1999; McCue and Shetty 2002a).

Some of the chemical elicitors studied caused an increase in total seed weight as compared to water controls (Figure 6C). A few examples include 8 d fava bean hypocotyl (46% higher) and wheat (27% higher) exposed to sucrose, and 8 d wheat exposed to gellan gum (33% higher). Due to increases in total phenolics per seed of most samples increasing in weight after elicitor exposure, it is possible that a bigger and heavier seed will need a higher phenolic content for serving protection and structure-giving functions proportional to seed volume and surface area. Increases in seed weight could be related to elicitor-enhanced activation of indole-3-acetic acid (IAA), a plant hormone playing an important role in the promotion of seedling growth (Chen and others 2002).

Most of the enhancements in phenolic contents and seed weight caused by elicitors were observed at late germination stages (days 5 to 8 after imbibition), rather than at earlier stages (day 2 after imbibition). Usually enzymes related to phenolic synthesis get activated within 24 h of elicitor treatment; however, differences in final phenolic synthesis of treatments start to show at day 3, 4, 5, or 6 after imbibition (Shetty, Atallah and Shetty 2001; McCue and Shetty 2002b; Randhir and Shetty 2004). This could be due to a series of necessary preceding reactions for the synthesis of primary

metabolites (i.e. proteins, carbohydrates, lipids) and signal molecules (i.e. reactive oxygen species).

A dose response experiment was conducted on mungbean seeds exposed to chitosan concentrations ranging from 0.001% to 1% and no clear dose-response behavior was exhibited on the phenolic content PSB of 4 d old mungbean seedlings (Figure 7A). Furthermore, on DB there seemed to be a decrease with increasing chitosan levels (Figure 7B). It is possible that mungbean seedlings responded to the high chitosan levels by giving priority to functions other than the synthesis of phenolic compounds. It was observed that dry matter and fresh weight of mungbean seedlings increased with chitosan concentrations (Figure 7 legend).

Once sucrose was identified to be a treatment significantly enhancing phenolic synthesis on fava bean and wheat, it was applied to the rest of the seeds. Seeds showing a significant (p-value < 0.139) increase in phenolic synthesis and/or TAC in response to sucrose included radish (8% higher TAC on a WB, Figure 8B), wheat (33% higher phenolics PSB, Figure 8C) and kale (27% higher phenolics PSB, Figure 8C). In the case of kale there was a significant (p-value = 0.018) increase in weight with sucrose treatment as compared to the control (Figure 9A), thus masking the effects of phenolic concentration on a WB; therefore the importance of expressing results on PSB as well. A dose response experiment was conducted on mungbean seeds imbibed in sucrose concentrations ranging from 0.1% to 10% and no significant changes were determined on phenolic content PSB; however, there was a significant decrease in phenolic concentration on a DB as sucrose concentration increased (Figure 10), which could be due to prioritizing towards the synthesis of compounds other than phenolics. As with chitosan treatment, it was also observed that dry matter and fresh weight increased with increasing sucrose levels (Figure 10 legend). Since both chitosan and sucrose are carbohydrates, both can be hydrolyzed and their glucose units utilized as precursors for the synthesis of other important compounds, thus diluting phenolic concentrations. It has been shown that plants possess the enzymes for hydrolyzing chitosan (Hung and others 2002), thus yielding extra carbons which could be utilized for diverse plant

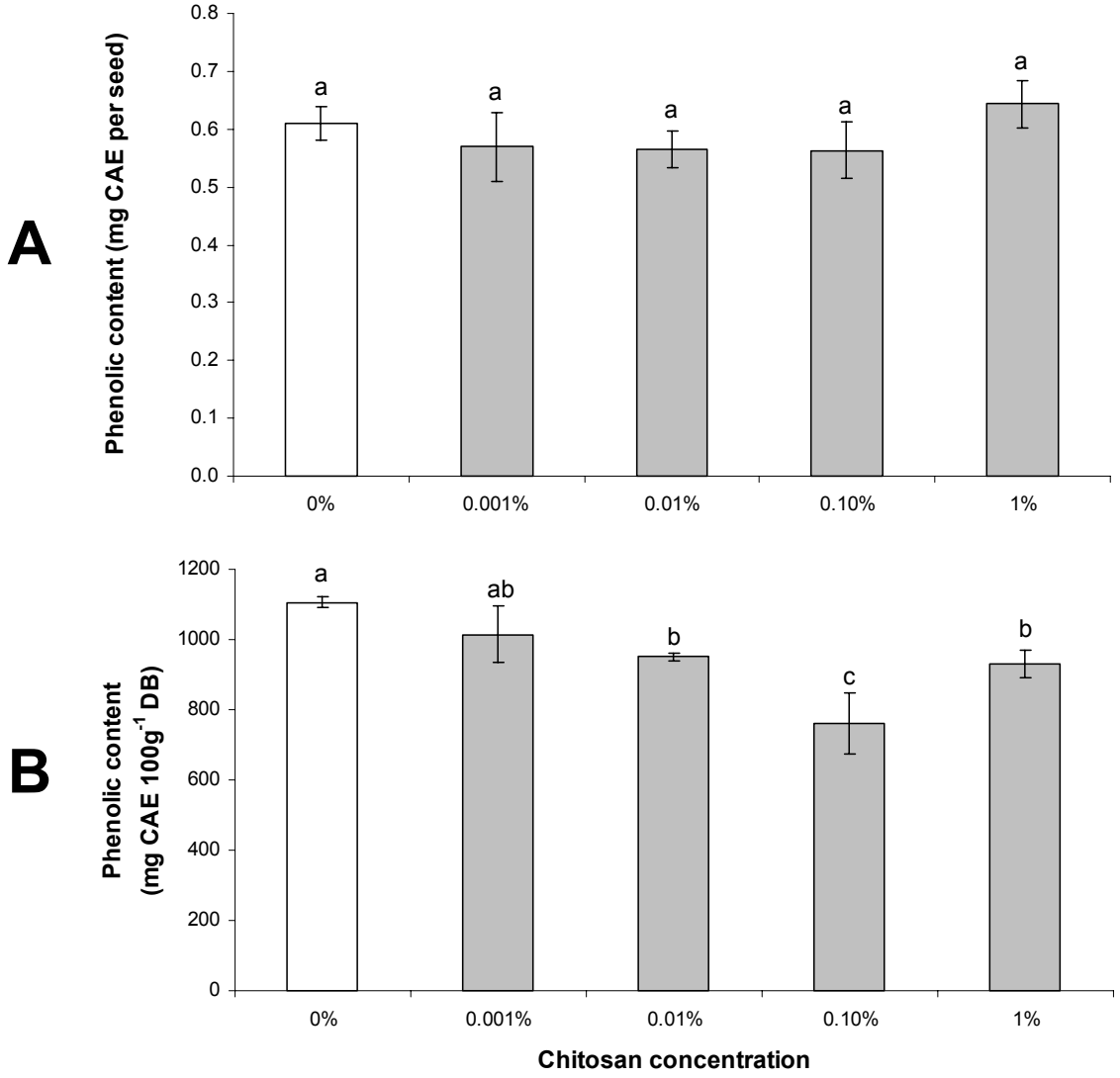

Figure 7 – Effect of increasing chitosan concentrations on phenolic content of 6 d old mungbean seedlings grown at 18°C. A: phenolic content per seed basis (PSB). B: phenolic content dry basis (DB). Dry matter/fresh weight per seed: 0% chitosan (15.2%/363mg), 0.001% chitosan (13.7%/409mg), 0.01% chitosan (15.5%/384mg), 0.1% chitosan (19.1%/388mg), 1% chitosan (16.4%/422mg). Columns within each figure with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Chitosan was dissolved in the imbibition solution. Bars show the average of 3 replicates ± standard deviation.

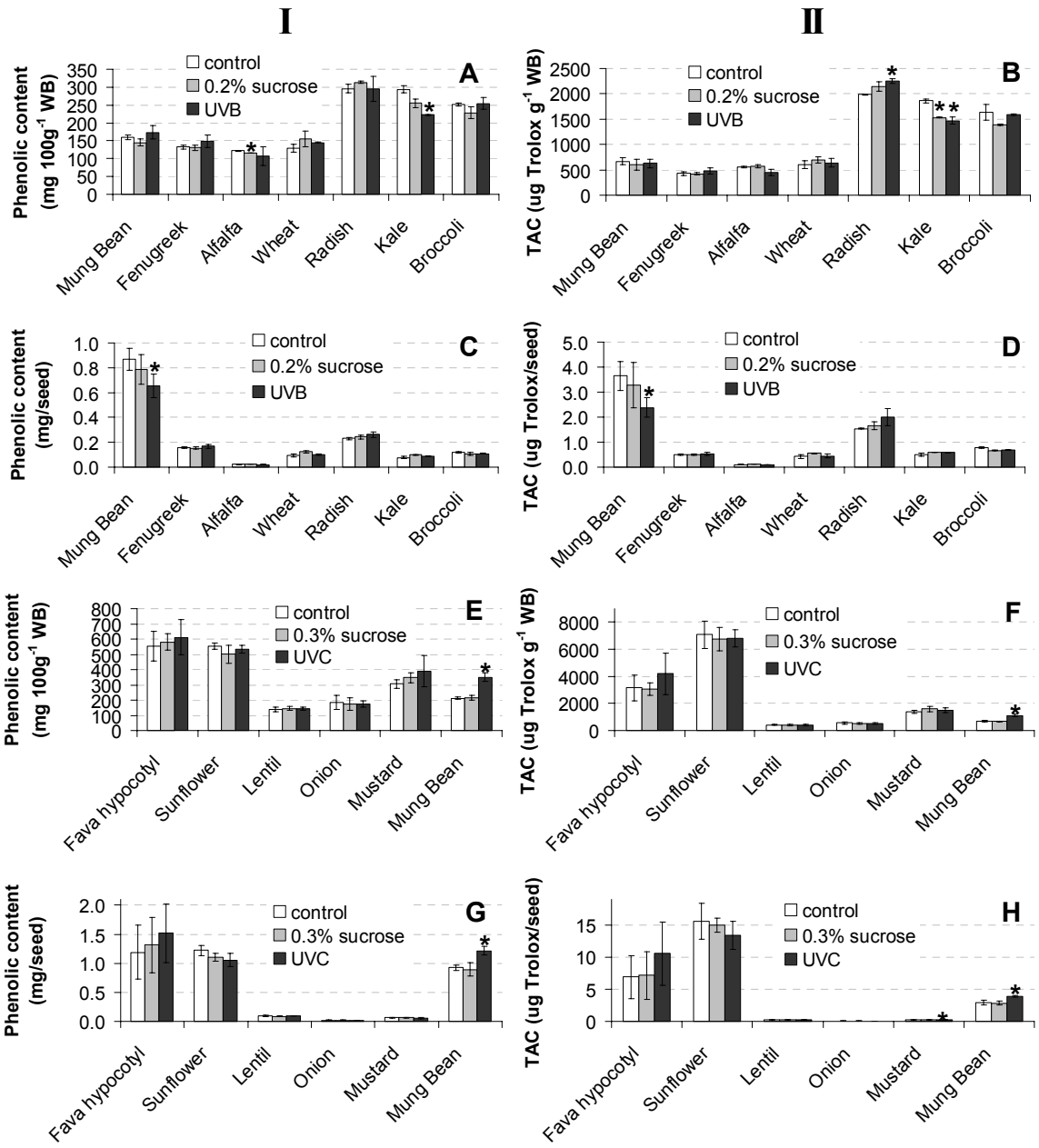

Figure 8 – Effect of sucrose and UV light (UV-B or UV-C) on phenolic content (I) and TAC (II) of 7 d seed sprouts grown at 18 °C. Insert figures A, B, E, F: wet basis (WB). Insert figures C, D, G, H: per seed basis (PSB). *Significantly different from control (α=0.05 with Duncan test). Sucrose was dissolved in the imbibition solution. UV-B and UV-C were applied 3 days after imbibition. Bars show the average of 3 to 6 replicates ± standard deviation.

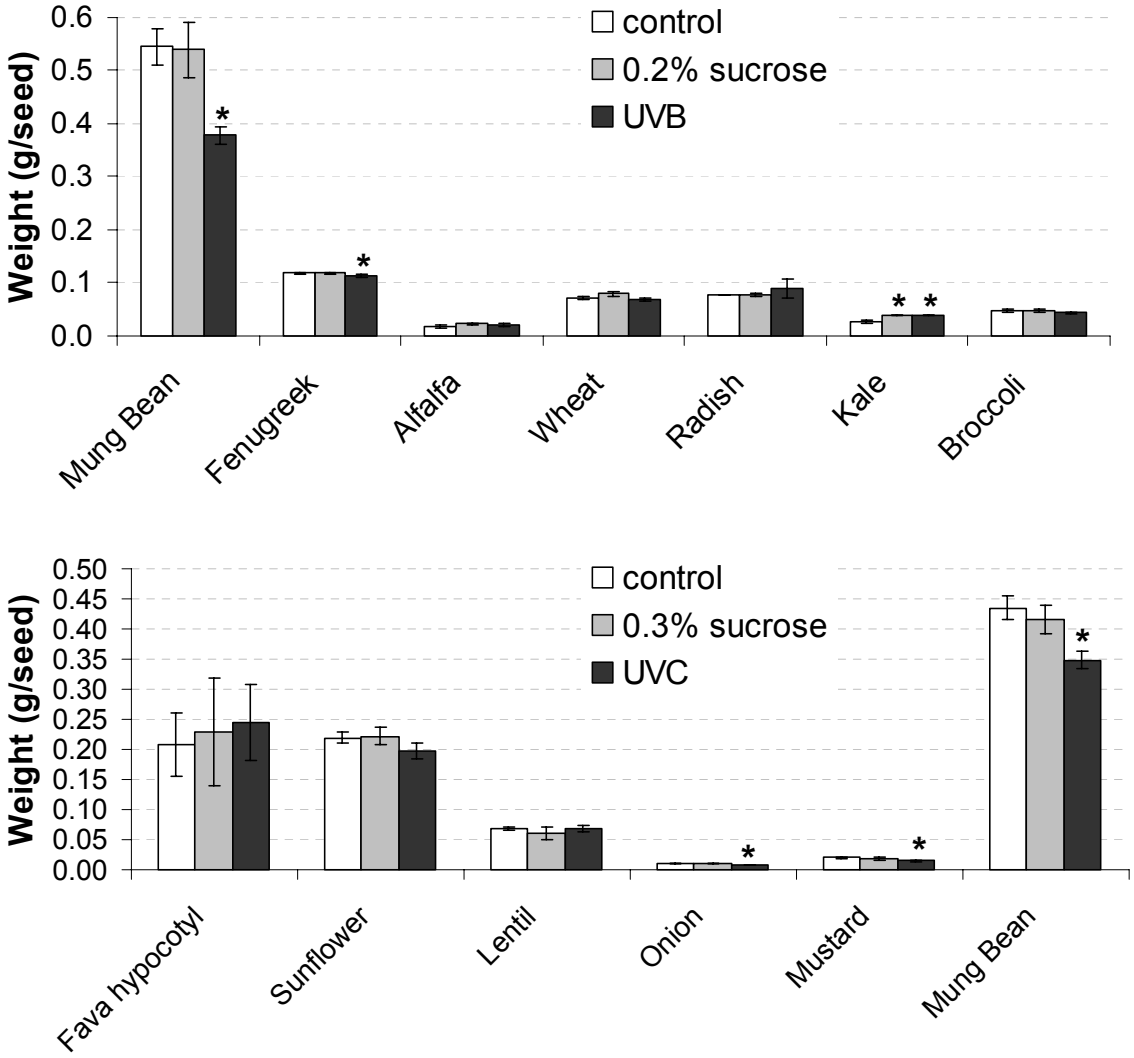

Figure 9 – Effect of sucrose and UV light (UV-B or UV-C) on fresh weight of 7 d seed sprouts grown at 18 °C. *Significantly different from control (α=0.05 with Duncan test). Sucrose was dissolved in the imbibition solution. UV-B and UV-C were applied 3 days after imbibition. Bars show the average of 3 to 6 replicates ± standard deviation.

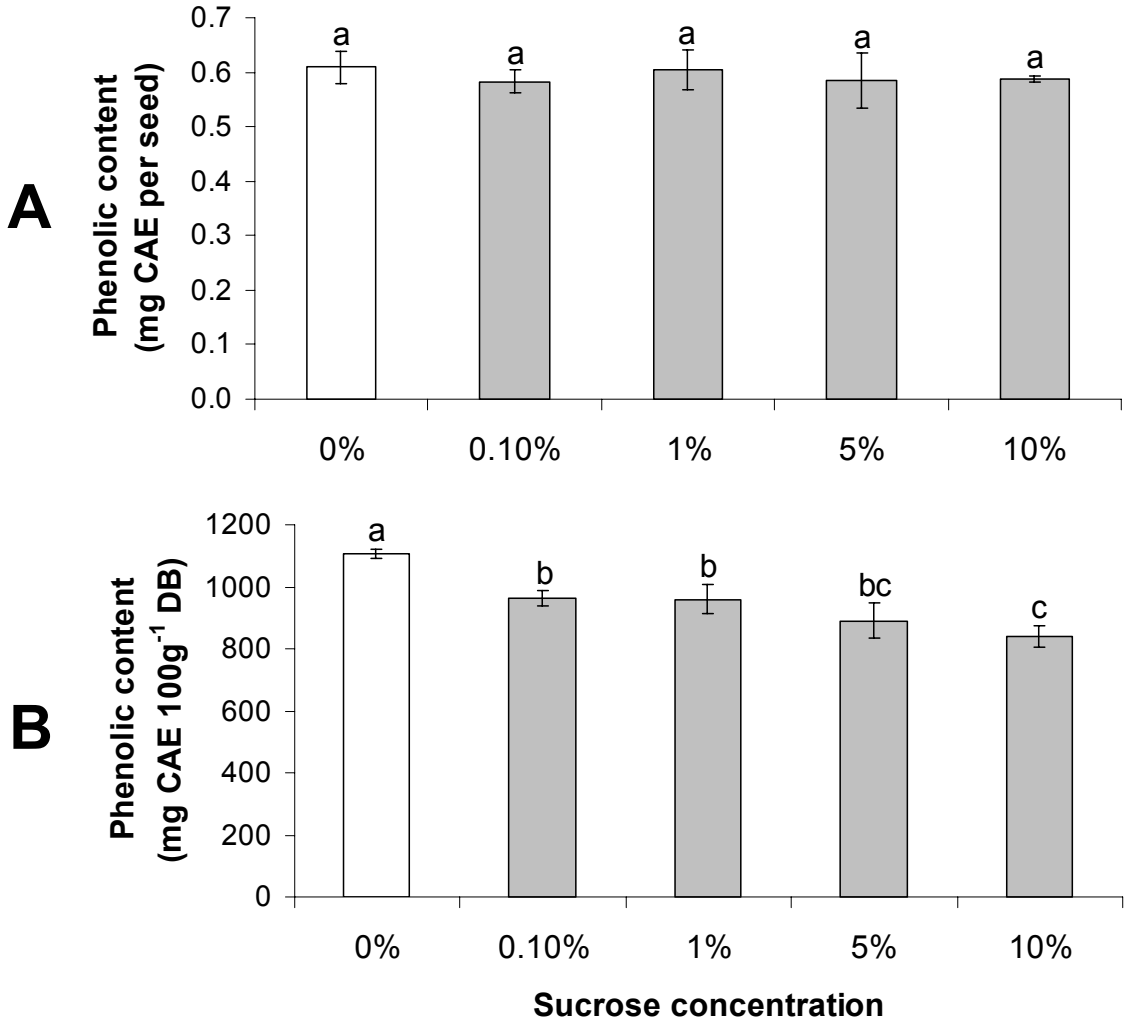

Figure 10 – Effect of increasing sucrose concentrations on phenolic content of 6 d old mungbean seedlings grown at 18 °C. A: phenolic content per seed basis (PSB). B: phenolic content dry basis (DB). Dry matter/fresh weight per seed: 0% sucrose (15.2%/363mg), 0.1% sucrose (15.2%/398mg), 1% sucrose (14.8%/427mg), 5% sucrose (15.1%/433mg), 10% sucrose (15.8%/443mg). Columns within each figure with similar letters are not significantly different from each other ($\alpha = 0.05$ with Duncan test). Sucrose was dissolved in the imbibition solution. Bars show the average of 3 to 6 replicates ± standard deviation.

metabolic functions. It is possible that sucrose and chitosan affected IAA activity, both compounds decreasing IAA activity in mungbean, whereas sucrose enhancing its activity in kale. Previous work has shown that the growth of Chinese foxglove root systems was enhanced by sucrose and chitosan applications (Hwang 2005).

The effect of UV light on phenolic antioxidants of 7 d sprouts

With regards to UV exposure, 7 d radish sprouts subjected to UV-B had 30% higher TAC PSB (p-value = 0.19) than controls with total phenolics increasing 13% (p-value = 0.18) (Figures 8C and 8D), while for fenugreek, both total phenolics (p-value = 0.13) and TAC on a WB (p-value = 0.19) increased 12% (Figures 8A and 8B). Differences in the antioxidant activity of phenolics synthesized in radish and fenugreek as response to UV-B could be due to a higher phenolic specific TAC of radish (Figure 3C), therefore showing higher increases in TAC than on phenolics. Upon UV-B exposure both radish and fenugreek responded by increasing phenolics; however phenolics from radish had a higher number of DPPH reactive hydroxyl groups.

With UV-C, mungbean and fava bean hypocotyl showed increases in total phenolics and TAC; however significant differences (p-value < 0.01) were observed only for mungbean (Figures 8E to 8H). For mungbean, increases in phenolics and TAC on a WB were ~66% (p-value = 0.001, Figures 8E and 8F), while increases in PSB were ~33% (p-value <0.01, Figures 8G and 8H). The lower values obtained on seed basis could be due to a 20% decrease in mungbean seed weight caused by UV-C treatment (Figure 9B). Increases in phenolics and TAC (WB and PSB) for fava bean hypocotyl ranged from 10% to 54%, but were not significant due to high variability (Figures 8E to 8H).

As with sucrose treatment, UV-B treatment on kale sprouts significantly (p-value = 0.015) increased fresh weight (50%, Figure 9A), decreasing phenolic content on a WB (-24% p-value = 0.009, Figure 8A) and slightly increasing phenolic content PSB (13% p-value = 0.25, Figure 8C). In relation to changes in seed weight due to UV, it has been previously shown that the plant growth hormone, IAA, is affected by UV and light

irradiation. Work by Chen and others (2002) showed that light reduces mungbean hypocotyl growth due to IAA inactivation by light activated peroxidases. Activation of IAA degrading peroxidases has also been observed in response to UV-B and UV-C (Murphy and Huerta 1990). Other researchers (Jayakumar and others 2003) observed that UV-A radiation promoted overall growth, while UV-B radiation inhibited development, with both UV sources causing reduced yields in dry matter, being the effect higher for UV-B. In another report (Pal and others 1999) UV-B caused 20% decrease in leaf area and 27% decrease in plant biomass, with increases in flavonoids and anthocyanins. In this last work, it was speculated that IAA photooxidation could have been involved (Pal and others 1999). The precise mechanisms of action of UV effect on growth and biomass in plants still needs to be elucidated.

A further experiment with mungbean seeds was conducted at different UV-C exposure times for confirming if previous enhancements will yield a dose-response behavior (Figure 11). Results showed that phenolic accumulation significantly increased with UV-C exposure time, thus verifying a positive dose-response relationship. Increases in phenolics on a DB were 4.2%, 21.2% and 42.3% for mungbean seeds exposed to 10, 20 and 40 min of UV-C radiation, respectively (Figure 11).

Phenolic increases in response to UV light could be due to the synthesis of antioxidant phenolics via a stress-induced response mechanism mediated by reactive oxygen species (ROS) activated by UV light (Frohnmeyer and Staiger 2003, Ros Barcelo and others 2003). Enzymes related to phenylpropanoid metabolism could get activated by ROS either for counteracting their oxidative damage or for exerting other protective functions such as lignification, or as UV-B filters. For low UV-B doses, as opposed to high energy UV-C, it is possible that specific UV-B receptors signal transcription towards phenolic synthesis, through production of key enzymes involved in flavonoid synthesis such as chalcone synthase (Frohnmeyer and Staiger 2003). Flavonoids are synthesized to act as UV-B shields thus protecting cellular DNA from dimerization and breakage (Dixon and Paiva 1995).

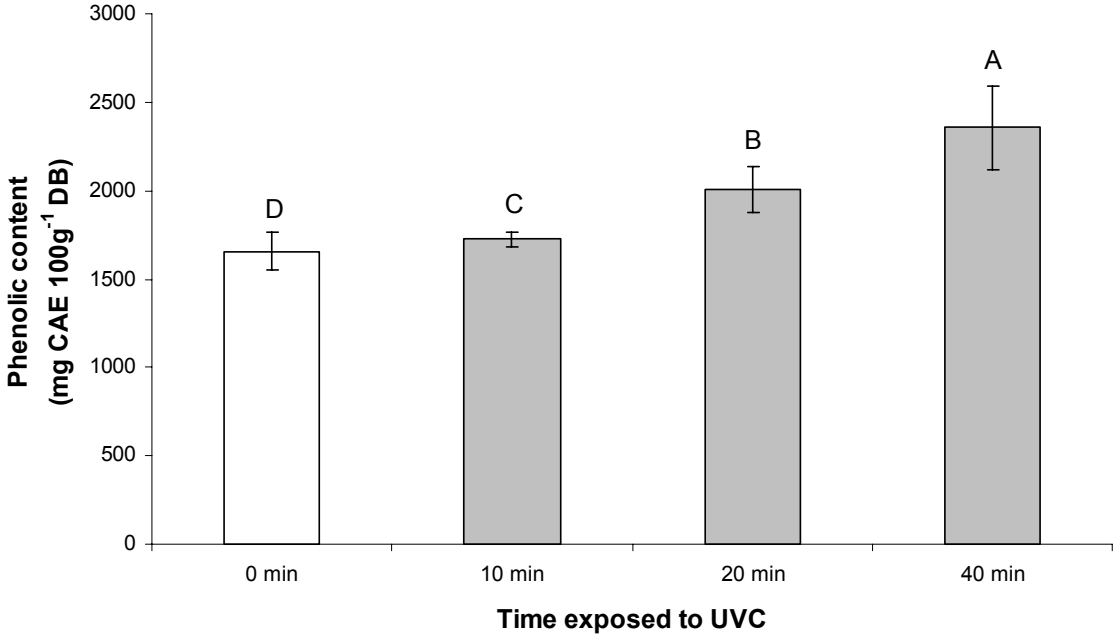

Figure 11 – Dose-response effect of UV-C on 7 d old mungbean seeds grown at 20 °C. Columns with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. UV-C was applied 3 days after imbibition. Bars show the average of 3 to 6 replicates ± standard deviation.

Seeds lacking a phenolic synthesis response to the tested chemical and physical elicitors, most likely were deficient in specific gene promoter and/or exonic sequences (Douglas and others 1991). In RNA synthesis, promoters are important for deciding which genes should be used for messenger RNA synthesis, thus controlling which proteins the cell manufactures. PAL and CHS genes seem to be expressed in response to environmental or developmental signals by having the correct gene promoter sequences; however, genes of other enzymes of the phenylpropanoid pathway such as 4-coumarate:CoA ligase (4CL) genes, need specific gene-internal sequences (exonic sequences), in addition to gene promoter sequences for responding to environmental signals, such as fungal elicitor and UV light (Douglas and others 1991). Other factors influencing plant respond to environmental signals include tissue specificity, developmental stage of the organism, metabolic or physiologic state of the cell and environmental signal level.

Conclusions

Simple tools for monitoring phenolic content and TAC of growing edible seeds allowed elucidating a few characteristics of the type of phenolic compounds being synthesized throughout growth and in response to chemical elicitors and UV light. We hypothesize that at initial germination stages phenolic compounds serve as antioxidants, while later they could become part of the structural framework of the growing plant and lose some of their antioxidant efficiency. When seeds were exposed to elicitors, sprouts increased the synthesis of phenolic compounds mostly for protecting the plant physically and chemically against environmental harm and predators as well as in response to an increase in carbon availability. Understanding how and why sprouts synthesize phenolics could help us obtain sprouts with enhanced nutraceutical levels and properties. For example, imbibition was shown to be one of the many ways to enhance the nutraceutical and commercial value of seeds. Potential applications of imbibed seeds might include production of nutraceutically enhanced seeds for animal feed, for value-added nutraceutical extracts, or for value-added seed by-products such as soybean

products (i.e. soy milk, soy sauce, tofu, okara) or malting products (i.e. beer, whisky). Innumerable commercial applications could be applied for mature sprouts as well. Sprouts could also serve as models for applying similar nutraceutical-enhancement strategies to other crops.

This chapter allowed the selection of mungbean as model for the following chapters. Mungbean was the seed that synthesized the greatest amount of phenolics during germination (based on initial levels) and after exposure to a physical elicitor (UV-C). In addition, due to its medium size, mungbean would ease the sampling process and help in the study of its sections (hypocotyl, cotyledon and seed coat) in relation to phenolic synthesis.

CHAPTER III

GERMINATION TEMPERATURE AND WOUNDING STRESS AFFECT THE PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF MUNGBEAN SEEDLINGS

Synopsis

Higher phenolic contents at higher germination temperatures were observed at the tested seedling growth stages. Two days after water imbibition, increases in phenolic content on a DB from dormant seed were 320%, 561%, 910% and 1137% for 10°C, 20°C, 32°C and 42°C, respectively. When wounding stress was applied to mungbean seeds, all wounding treatments (wounded after imbibition [0DW], wounded 2 days after imbibition [2DW] and wounded 4 days after imbibition [4DW]) yielded higher phenolic content and total antiradical capacity (TAC) than non-wounded controls. Non-wounded, 0DW, 2DW and 4DW mungbeans increased phenolics by 962%, 1094%, 1193% and 1032%, respectively, after 6 d at 18°C, compared to dormant seed. Wounding showed the greatest effect on phenolic synthesis after water imbibition (0DW), with a phenolic synthesis rate (R_{PS}) of 1.94 from day 0 to day 2 (R_{PS} for control was standardized at 1.00). Wounded seed treatments exposed to 25°C and 32°C enhanced phenolic content compared to controls only for two days following imbibition and thereafter declined due to possible phenolic esterification to the cell wall or cross-linking with lignin at later stages. The phenolic specific TAC showed that values for wounded seeds grown at 25 and 32°C were higher than controls throughout the tested growing period, suggesting the synthesis of phenolics with higher antioxidant activity. All wounded seeds possessed similar phenolic specific TAC, regardless of temperature, showing the synthesis of similar phenolic antioxidants.

Introduction

Temperature is a very important factor for seed germination. Some seeds require chilling temperatures (0 to 10°C) in a fully hydrated state in order to germinate (Taiz and

Zeiger 1998c), while seeds such as mungbean, a warm season annual pulse, have an optimum germination and growth temperature range of 27-30°C (Imrie 2005). This temperature range might be optimum for germination and elongation of mungbean; however little is known about optimum temperatures for the synthesis of phenolic antioxidants in germinating seeds. In previous studies, phenolics have been monitored in germinating seeds but at a single temperature (Canella and Castriotta 1982, McCue and Shetty 2002a, Shetty and others 2003, Wang and others 2005). For other plant tissues, temperature studies related to phenolics have been conducted in relation to anthocyanin synthesis and chilling injury prevention (Chalker-Scott L 1999, Reay 1999, Janas and others 2000, Saltveit 2002, Lukatkin 2005). A very recent study has been conducted at several temperatures monitoring phenolics; however this work was on somatic embryogenesis and compound synthesis optimization for applications in bioreactors (Shohael and others 2006). Their conclusions were that somatic embryos are very sensitive to low (12 and 18°C) and high (30°C) temperatures and have a decreased phenolic synthesis at these extreme temperatures. To our best knowledge, no work on temperature studies has been conducted on the enhancement of phenolics with antioxidant activity in edible seedlings.

Temperature is crucial to phenolic synthesis, since these metabolites are catalyzed by enzymes and enzyme-catalyzed reactions are temperature dependent. Most of these enzymatic reactions show an exponential increase in rate with increasing temperature, followed by a decrease in activity when protein denaturation starts (Taiz and Zeiger 1998d). Usually protein denaturation begins around 40 to 50°C and is complete over a range of ~10°C (Taiz and Zeiger 1998d). It is also possible that at temperature extremes (4°C or 40°C), production of reactive oxygen species (ROS) is enhanced, therefore increasing signaling of phenylpropanoids catalyzed by phenylalanine ammonia lyase (PAL) (Dat and others 2000). As there are enzymes that synthesize phenolics (i.e. PAL), there are other enzymes responsible for transforming and degrading them (i.e. peroxidase [POX], polyphenol oxidase [PPO], cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase [CAD]) (Douglas and others 1992). A

positive balance on phenolic content at a given germination stage would indicate that phenolic synthesis rates were higher than phenolic transformation/degradation rates.

Wounding is associated with changes in phenolic content, and results on several plant tissues, including fruits and vegetables, have shown that wounded tissues of some plant species have higher phenolic content and antioxidant capacity than non-wounded controls (Toivonen and DeEll 2002; Heredia and Cisneros-Zevallos 2002; Kang and Saltveit 2002; Reyes and Cisneros-Zevallos 2003). When plants are subjected to wounding stress, stress-related defense genes are activated to accomplish functions such as to neutralize the stress, repair damage, synthesize compounds to inhibit growth of predators, activate defense signaling pathways and adjust the plant metabolism to the nutritional demands (Dat and others 2000, Leon and others 2001; Stratman 2003). Upon wounding stress, several enzymes related to phenolic synthesis may be activated such as the first enzyme of the shikimate pathway, 3-deoxyarabinoheptulosonate phosphate synthase (DAHP synthase), and the first enzyme of the phenylpropanoid pathway, PAL (Hrazdina 1992).

The objective of this chapter was to study phenolic synthesis and antioxidant activity at different germination temperatures (10°C to 42°C), in response to wounding stress applied at different germination stages. The stages considered are: immediately after imbibition [0DW], 2 days after imbibition [2DW] and 4 days after imbibition [4DW]).

Materials and Methods

Materials

Mung bean (*Vigna radiata* L. Wilczek) seeds were purchased from Johnny's Selected Seeds (Winslow, ME, USA). Chlorogenic acid, Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Seed germination

Mungbean seeds were allowed to imbibe water at different temperatures (10°C, 18°C, 20°C, 25°C, 32°C and 42°C) for 14 h. Then water was removed and seeds were dark-germinated in glass jars with 3 layers of humidified paper towels at the same temperatures on which they were imbibed. Paper towels were kept moist by spraying with sterile water as needed.

Seeds were assayed for dry matter, total phenolics and total antiradical capacity (TAC) through time at the different experimental conditions.

Wounding and temperature experiments

Non-wounded mungbean seeds were grown at 10°C, 18°C, 20°C, 32°C and 42°C for 8 days. Total phenolics and TAC were measured at day 0, 2, 4, 6 and 8. Some seeds were transferred from one growing temperature to another for determining the effect of chilling on phenolic synthesis. Some seeds grown at 10°C were transferred to 32°C at day 6, while some seeds grown at 32°C were transferred to 4°C at day 2 and then transferred back to 32°C at day 6.

For wounding treatment, mungbean seeds were wounded at day 0 (water imbibed stage [0DW]), day 2 (2DW) and day 4 (4DW). Total phenolics and TAC were evaluated at days 0, 2, 4 and 6. Wounding was applied with a sterile razor blade and the seed was completely cut on both the longitudinal and latitudinal axis, to yield four sections. These seeds were grown at 18°C.

Calculations of phenolic synthesis rates (R_{PS}) of wounded treatments compared to non-wounded controls were as follows:

$$R_{PS\ t1\text{-}t2} = \frac{(\text{Phenolic content wounded}_{\ \text{time 2}} - \text{Phenolic content wounded}_{\ \text{time 1}})}{(\text{Phenolic content control}_{\ \text{time 2}} - \text{Phenolic content control}_{\ \text{time 1}})}$$

This parameter, R_{PS}, gives an estimate of phenolic synthesis, when compared to a control; however, it only shows the resulting balance between real phenolic synthesis rates and phenolic transformation/degradation rates.

For the combination of wounding and germination temperature, day 0 non-wounded and wounded mungbean seeds were grown at temperatures of 18°C, 25°C and 32°C for 6 days. Total phenolics and TAC were measured at days 0, 2, 4 and 6.

For all assays, 6 seeds were used per replicate, with 4 to 8 replicates per assay and 3 to 6 repetitions per replicate.

Total soluble phenolics

Total soluble phenolic content of methanolic extracts was assayed as described by Cevallos-Casals and Cisneros-Zevallos (2003) but adapted for microtiter plate reader measurements. Thirteen uL of methanolic sample extracts (equivalent volume of methanol for the blank) were loaded in each well of a 96-well flat bottom microtiter plate (Costar #3595, Corning, Inc., Corning, NY). Using a multi-channel micropipette, 208 µL of nanopure water were added to each well followed by 13 µL of Folin-Ciocalteu reagent. Mixture was allowed to react for 3 min, after which 26 µL of 1 N Na_2CO_3 were added. Plates were sealed with one layer of parafilm, allowed to react for 2 h, and absorbance read at 725 nm in a Synergy HT microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Total phenolics were expressed as mg chlorogenic acid equivalents (CAE) 100 g^{-1} wet basis (WB) or dry basis (DB), based on a standard curve.

Total antiradical capacity (TAC)

TAC of phenolic compounds was adapted from Brand-Williams, Cuvelier, and Berset (1995) to be used in a microtiter plate reader. Thirteen uL of methanolic sample extract (equivalent volume of methanol for the blank) were mixed with 247 µL of DPPH solution (98.9 uM in methanol) inside each well of a 96-well flat bottom microtiter plate (Costar #3595, Corning, Inc., Corning, NY). Plates were tightly sealed with several

layers of parafilm to prevent evaporation, placed in the dark at 20 °C for 20 h and read at 515 nm in a Synergy HT microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The change in absorbance was used and results were expressed as ug Trolox equivalents g^{-1} WB or DB, from a standard curve. In addition, specific antioxidant capacity (specific TAC) was defined as the ratio of total antiradical capacity/total soluble phenolics and expressed as μg Trolox equivalents mg^{-1} CAE. The specific antioxidant capacity provides information on the effectiveness of phenolics to neutralize free radicals. A higher specific TAC means phenolic compounds have a higher capacity to stabilize free radicals.

Analysis of variance and covariance

One-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Means were compared with Duncan's Multiple Range Test at $\alpha = 0.01$ and 0.05.

Results and Discussion

Effect of germination temperature on the phenolic content and antioxidant activity of mungbean seeds

During 14 h water imbibition at different temperatures there was a high increase in phenolic content as was observed in Chapter I. Increases in phenolic content on a DB from dormant seed (phenolic content 49.1 mg CAE $100g^{-1}$) to 14 h water imbibed seed were 253%, 262%, 217% and 157% at 10°C, 20°C, 32°C and 42°C, respectively. At this stage lower imbibition temperatures yielded seeds with higher phenolic contents; however at later germination stages phenolic synthesis increased proportionally with germination temperature (Figure 12). Lower phenolic contents of imbibed seeds at higher temperatures could be explained by a higher exudation of synthesized phenolic compounds through the plant cell wall and membrane. Seeds imbibed at higher temperatures absorbed more water, which could be due to higher cell wall and membrane permeability, thus facilitating liquid and solute interchanges. Plasma

membrane lipid conversion from gel to fluid could have progressed together with increases in temperature, with the membrane becoming too fluid to maintain the permeability barrier (Staehelin and Newcomb 2001). As a result of water imbibition, solutes and low-molecular-mass metabolites could have leaked from the gel-like or liquid-crystalline-like plasma membrane (Bewley and others 2001). Shortly after imbibition, the stable liquid-crystalline arrangement is resumed, and leakage could have stopped (Bewley and others 2001).

Two days after water imbibition, increases in phenolic content on a DB from dormant seed were 320%, 561%, 910% and 1137% for 10°C, 20°C, 32°C and 42°C, respectively (Figure 12). Higher increases in phenolic contents at higher germination temperatures were observed throughout the tested growth days. For example at day 8, increases in phenolic content on a DB from dormant seed were 1794% and 2552% for 20°C and 32°C, respectively. Linear regressions ($R^2 > 0.98$) were fitted through phenolic content data for seeds germinated at 10°C, 20°C, 32°C and 42°C versus time; and results showed that all slopes were statistically different at $\alpha = 0.01$ using ANCOVA (testing for slopes), indicating that temperature is a significant factor (covariate) affecting phenolic synthesis. Even though seeds exposed to 42°C experienced the highest increase in phenolic content at day 2, 42°C experiments were stopped at this time due to initial signs of seed spoilage.

The effect of temperature changes in phenolic synthesis was also examined. Some seeds grown at 10°C were transferred to 32°C at day 6, while some seeds grown at 32°C were transferred to 4°C at day 2 and then transferred back to 32°C at day 6 (Figure 12). Seeds grown at 10°C more than doubled their phenolic content when taken to 32°C for 2 days. Seeds grown at 32°C ceased their phenolic synthesis when they were transferred to 4°C; however synthesis continued at the expected rate (slope) when seeds were transferred back to 32°C. Phenolic synthesis rates from day 6 to day 8 for seeds grown at 32°C, for seeds grown at 10°C, then transferred to 32°C, and for those grown at 32°C, chilled to 4°C for 4 days and then transferred back to 32°C, were very similar; indicating that synthesis rates were not affected by previous exposure to 4°C or 10°C.

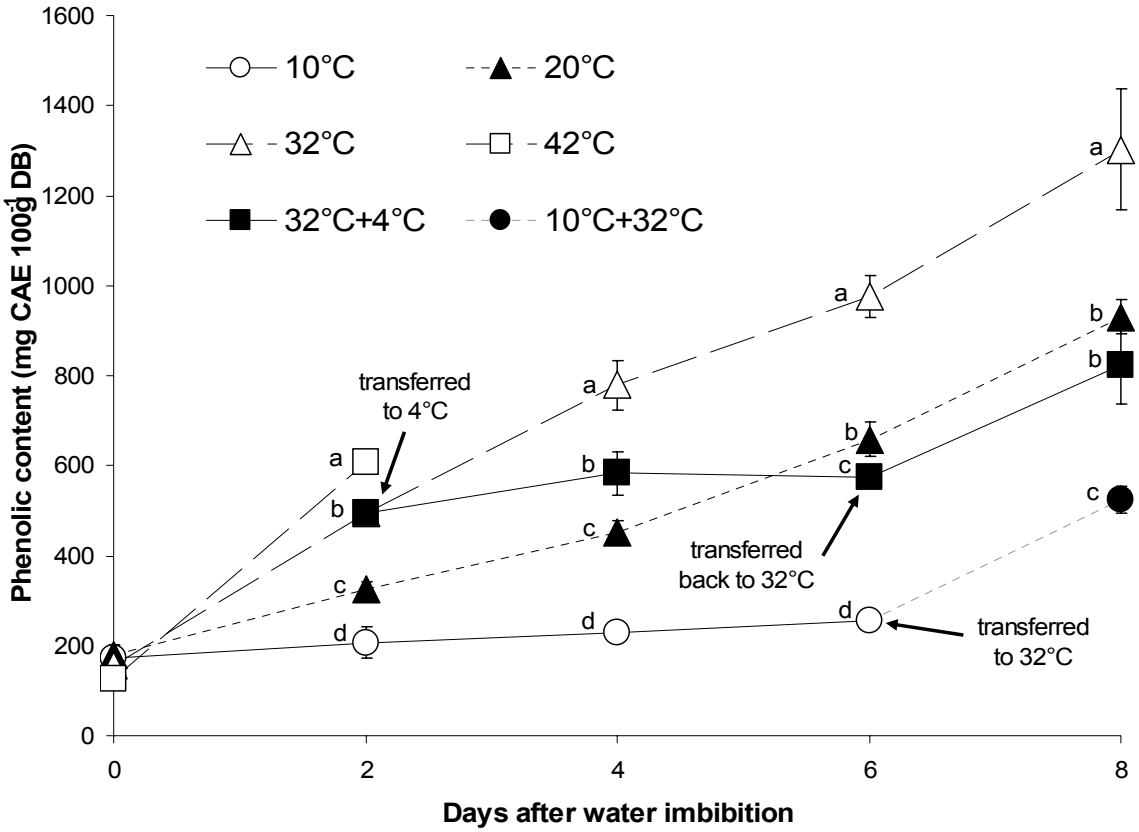

Figure 12 – Phenolic content of mungbean seeds at different germination temperatures. Arrows indicate transfer of seeds from one temperature to another. Similar letters within the same germination time are not significantly different (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

These results contradict some studies that show that cold stress increases the amount of soluble phenolics (Janas and others 2000, Janas and others 2002). It is possible that cell wall bound phenolics or specific phenolic compounds, not quantified in our study, could have increased due to chilling stress. For example, some studies have shown increases in specific phenolic acids and isoflavonoids in soy bean roots (Janas and others 2002, Pennycooke and others 2005). Regarding mungbean responses to chilling stress, previous work has shown increases in reactive oxygen species, antioxidant enzymes and other elements related to plant protection during chilling (Yu and others 2003).

For TAC, results showed similar trends as for soluble phenolic contents, higher antioxidant activity as germination temperature increased (Figure 13). For example at day 6, increases of TAC on a DB from dormant seed (TAC 225.2 ug Trolox g^{-1}) were 440%, 630% and 1297% for 10°C, 20°C and 32°C, respectively. Increases at day 8 were 1101% and 1809% for 20°C and 32°C, respectively.

These results indicate that the enzymes related to phenylpropanoid metabolism (i.e. phenylalanine ammonia lyase [PAL], chalcone synthase) may be synthesized and/or activated at rates proportional to increases in temperature from 10°C to 42°C. These increases in enzymatic activity could be in part due to optimum enzyme-catalyzed reaction temperatures and/or mediated by reactive oxygen species (ROS), which may accumulate even more at higher temperatures (Dat and others 2000, Shohael and others 2006). In addition, it is possible that phenolic oxidizing or related enzymes such as polyphenol oxidase and/or catalase are inactivated at these high temperatures. In the case of catalase, its inactivation would inhibit the conversion of H_2O_2 to H_2O and higher H_2O_2 levels could elicit higher synthesis of phenolic compounds. Work has shown catalase to be inactivated at relatively mild temperatures. For example, *N. plumbaginifolia* during a 5 h heat shock at 37°C, showed lower catalase transcript levels than at lower temperatures (Dat and others 2000). Decreases in catalase have also been observed with temperature increases from 12 to 30°C in somatic embryos of *Eleutherococcus senticosus* (Shohael and others 2006).

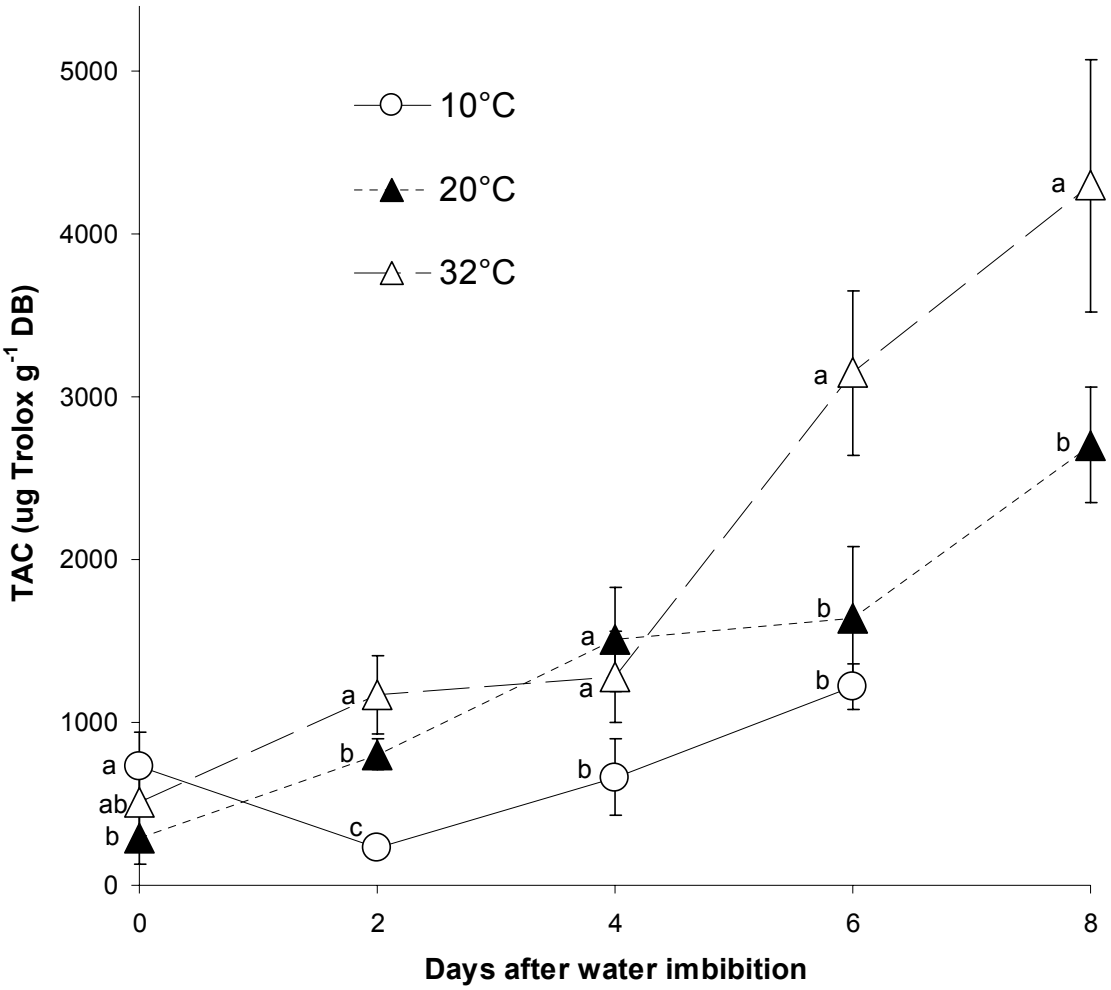

Figure 13 – TAC of mungbean seeds at different germination temperatures. Similar letters within the same germination time are not significantly different (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

Effect of wounding stress, applied at different germination stages, on the phenolic content and antioxidant activity of mungbean seeds

When wounding was applied to mungbean seeds, all wounding treatments (0DW, 2DW and 4DW) enhanced the phenolic synthesis expressed on a WB and DB (Figure 14). Since phenolic content on a WB changed due to water absorption, results were discussed based on a DB (Figure 14B). Non-wounded seeds, 0DW, 2DW and 4DW mungbean seedlings increased phenolics by 962%, 1094%, 1193% and 1032%, respectively, after 6 d at 18°C, compared to levels found in dormant seed. The greatest effect on phenolic synthesis occurred with wounding applied after water imbibition (0DW), with a phenolic synthesis rate (R_{PS}) of 1.94 from day 0 to day 2 (R_{PS} for non-wounded controls were standardized at 1.00). The other wounding treatments applied at later times elicited phenolic synthesis but at lower synthesis rates (2DW: $R_{PS\ d2\text{-}d4} = 1.61$; 4DW: $R_{PS\ d4\text{-}d6} = 1.29$) (Figure 14). Interestingly, the higher initial phenolic synthesis rates observed for 0DW, decreased as germination progressed ($R_{PS\ d0\text{-}d2} = 1.94$, $R_{PS\ d2\text{-}d4} = 1.21$, $R_{PS\ d4\text{-}d6} = 0.45$). It is possible that phenolic transformation/degradation rates increased with germination time, thus showing a net result of decreasing R_{PS}. For example, soluble phenolics could have been esterified to the cell wall and subsequently incorporated into lignin or suberin, thus decreasing the overall soluble phenolic content.

Results for TAC showed similar trends as for total phenolics, indicating an enhanced synthesis of phenolic antioxidants due to wounding applied at different germination stages (Figure 15). Differences in TAC between wounded treatments and control were significant (p-value < 0.05) for 0DW at day 2 and for 2DW at days 4 and 6.

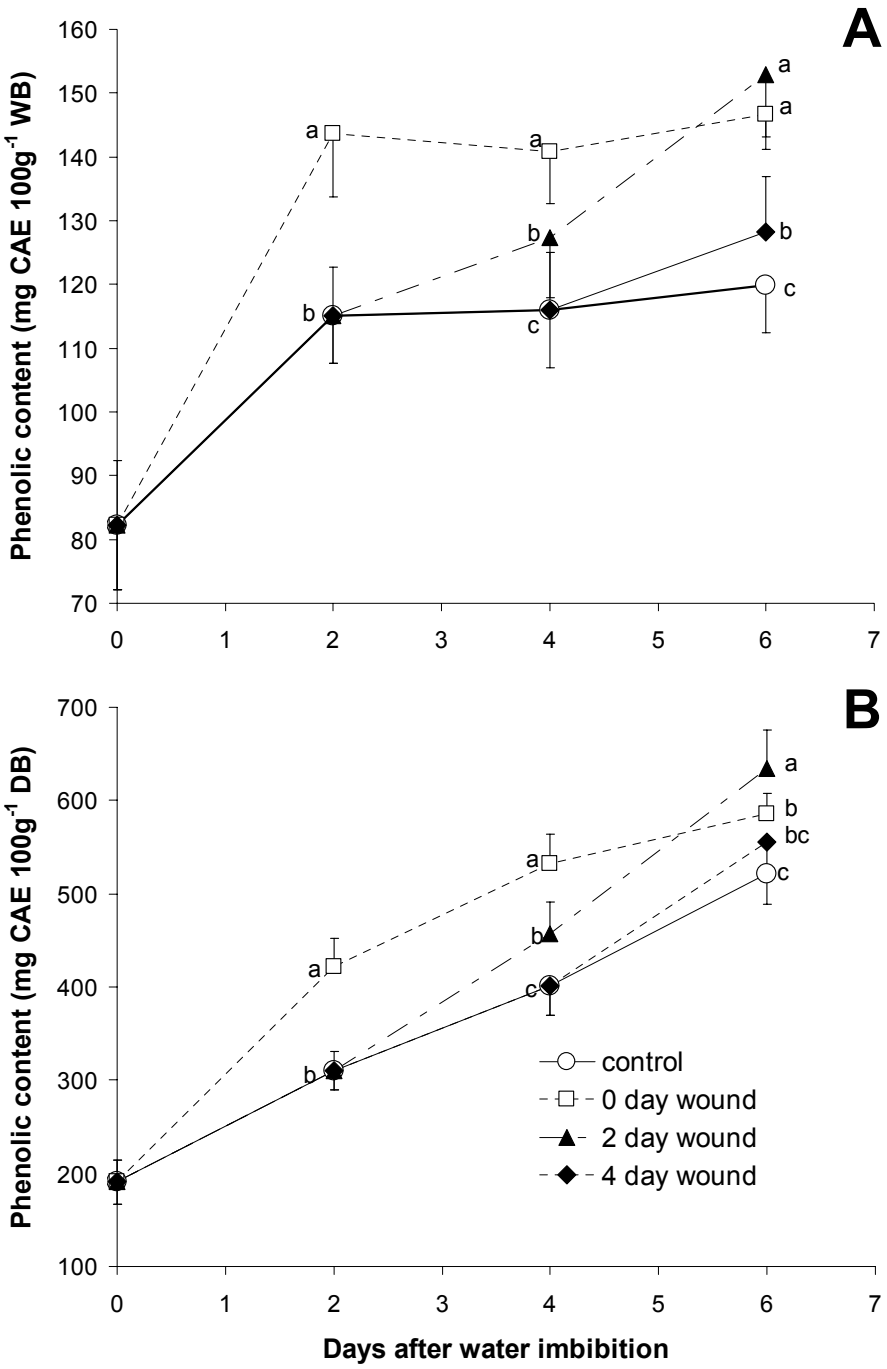

Figure 14 – Phenolic content of mungbean seeds wounded at different germination stages grown at 18°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. A: phenolic content expressed on a wet basis; B: phenolic content expressed on a dry basis. Data shows the average ± standard deviation, n = 8.

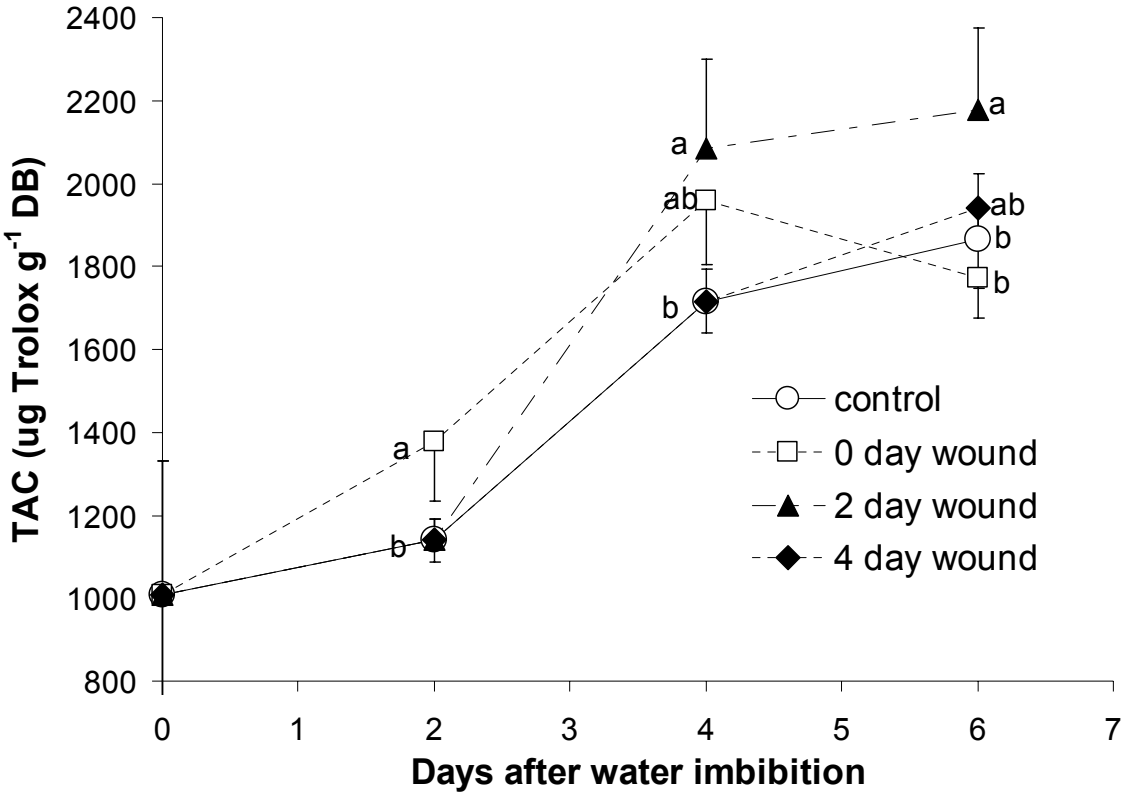

Figure 15 – TAC of mungbean seeds wounded at different germination stages grown at 18°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

Effect of wounding stress, at different germination temperatures, on the phenolic content and antioxidant activity of mungbean seeds

When wounded seeds were grown at different temperatures, it was hypothesized that higher germination temperatures would have an enhanced effect on soluble phenolic synthesis of wounded mungbean seeds, either additive or synergistic. However, results shown in Figure 16 indicate that wounding treatments exposed to 25°C and 32°C did enhanced phenolic content the initial two days after imbibition, after which the phenolic accumulation rate declined. It is likely that phenolic transformation/degradation rates started to become higher than phenolic synthesis rates at later germination stages (day 4 and above) for temperatures ≥ 25°C. The observed decline in phenolic synthesis at temperatures of 25°C and 32°C could be due to possible lignification process favored in these wounded tissues. Thus, the initial transient increase in soluble phenolic compounds indicates soluble phenolic transformation into precursors for lignin and suberin, considered important compounds for plant protection and structure (Douglas and others 1992). In addition, possible increases in ROS (i.e. H_2O_2) due to high temperatures, could have also mediated enhanced activity and/or gene expression of peroxidases, which help oxidize phenolics into lignin in the presence of H_2O_2 (Passardi and others 2005). On the other hand, the same effect observed at temperatures ≥ 25°C was not present for wounded seeds grown at 18°C; however, it is likely that it will occur at later growth stages (≥ 7 d).

With regards to TAC, results showed similar trends as for soluble phenolic content for non-wounded controls only (Figure 17). TAC results for wounded seeds at 25°C and 32°C were higher than controls at days 2 and 4 after imbibition. When calculating TAC based on phenolic basis, we observed that the phenolic specific TAC of wounded seeds became higher than controls at 25°C and 32°C, but not at 18°C (Figure 18), suggesting the synthesis of phenolics with a greater number of DPPH reactive OH groups. Even though there is degradation/transformation of soluble phenolics in wounded tissues at higher temperatures, there seems to be signaling towards the synthesis of phenolics with high antioxidant activity for possible functions such as

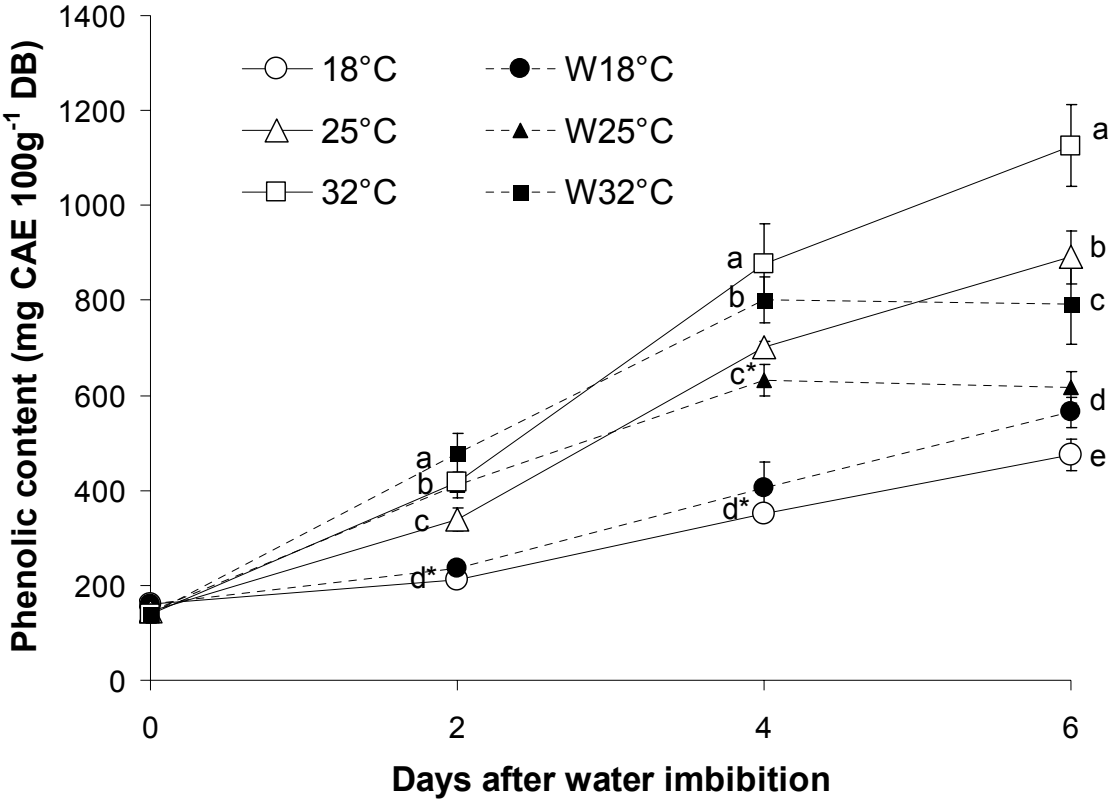

Figure 16 – Phenolic content of wounded and non-wounded mungbean seeds grown at three different germination temperatures. Open symbols represent non-wounded controls, while dark symbols represent wounded seeds. Wounding for all treatments was done after imbibition (0DW). Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Letters accompanied by * indicate wounding to be significantly different from control ($\alpha = 0.05$ with Duncan test) when evaluated independently. Data shows the average ± standard deviation, n = 4.

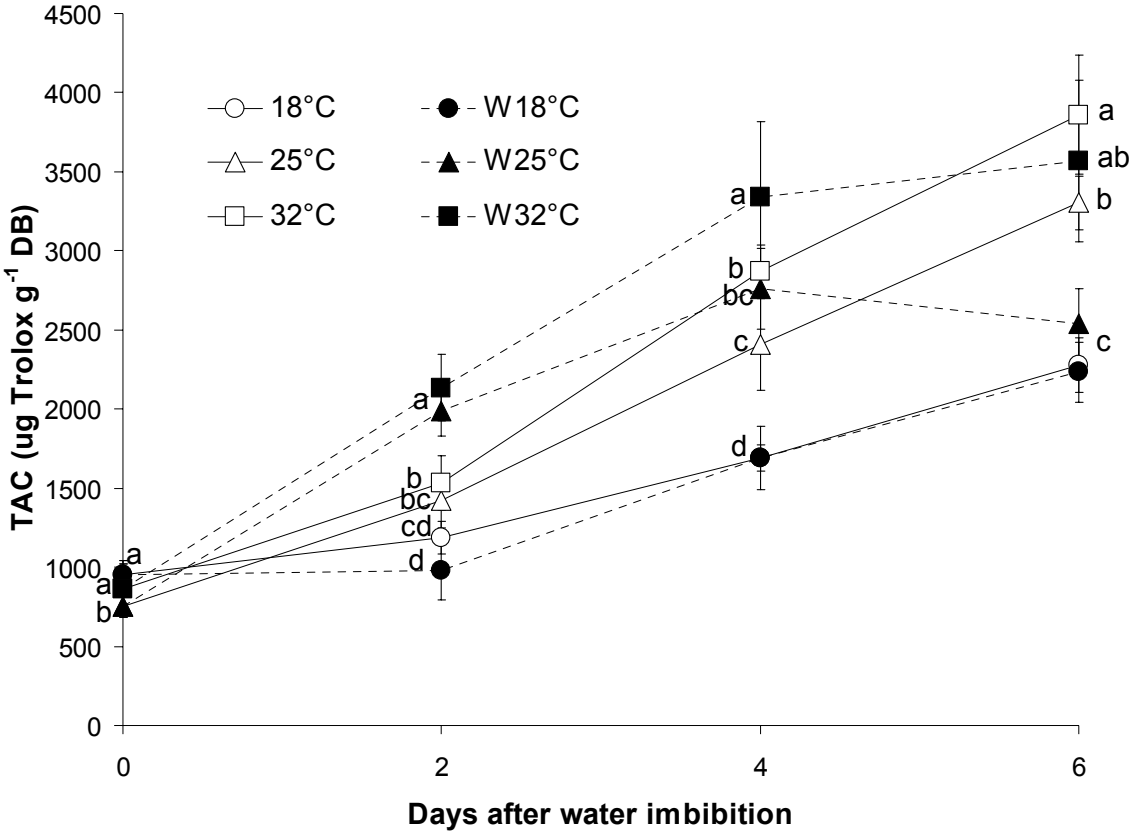

Figure 17 – TAC of wounded and non-wounded mungbean seeds grown at three different germination temperatures. Open symbols represent non-wounded controls, while dark symbols represent wounded seeds. Wounding for all treatments was done after imbibition (0DW). Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

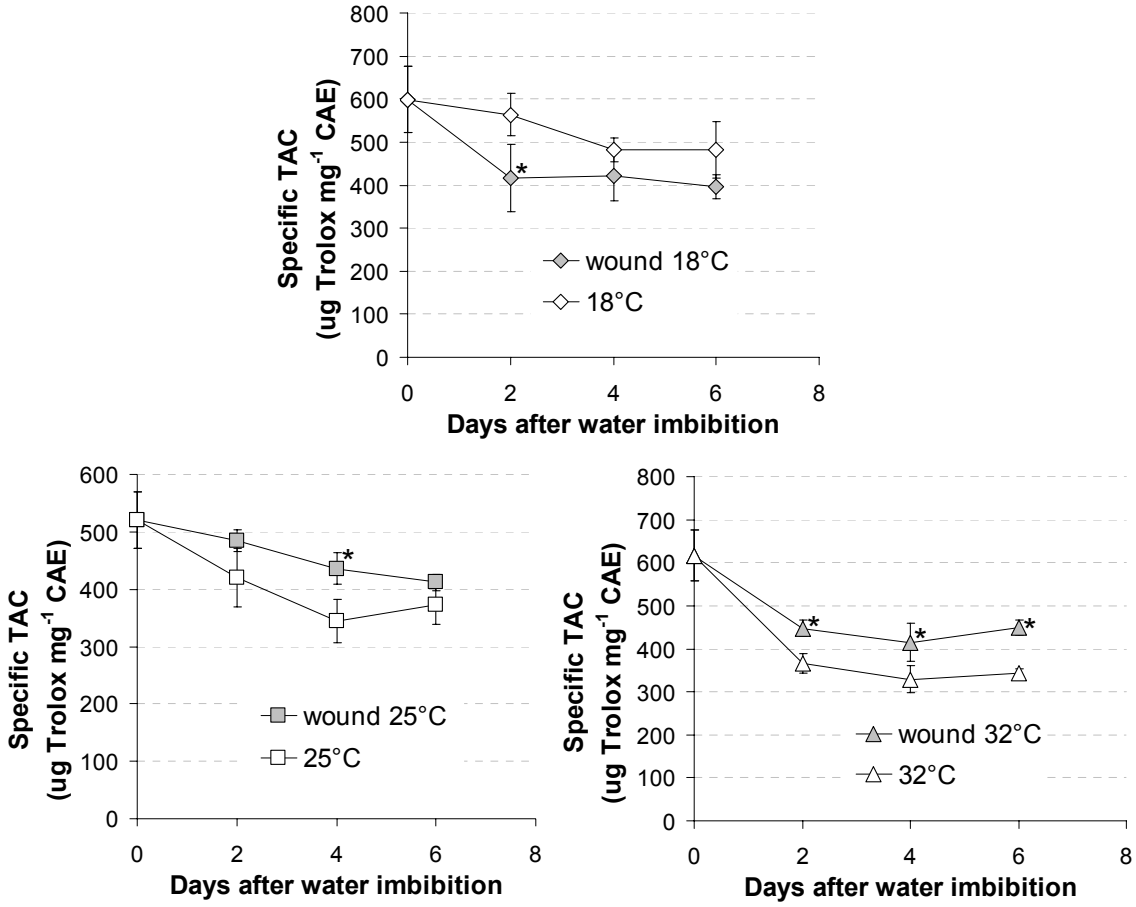

Figure 18 – Changes in phenolic specific TAC for wounded and non-wounded mungbean seeds grown at 18°C, 25°C and 32°C. Significant differences ($\alpha = 0.05$ with Duncan test) of wounding respect to control at the same germination time are shown with *. Data shows the average ± standard deviation, n = 4.

counteracting evolution of reactive oxygen species (ROS), which tend to be favored by higher temperatures and signals of stress. From Figure 18 data it was observed that phenolic specific TAC tended to decrease as germination progressed. This decrease in specific TAC reinforces results from Chapter II, on which phenolic specific TAC of 7 d old sprouts was lower than that of imbibed seeds, suggesting a higher need of phenolic antioxidants during early germination stages (i.e. water imbibition stage) than at later germination stages. It was also observed that the phenolic specific TAC for wounded tissues at the tested temperatures was similar for all germination stages (Figure 18). This shows that regardless of temperature, wounding stress signals the synthesis of phenolic compounds with similar antioxidant activity.

Conclusions

This Chapter allowed a better understanding of germination temperature and wounding effects on the synthesis of phenolic compounds and their antioxidant properties in growing seedlings. Wounding and temperature can be combined to enhance the amount of phenolic antioxidants; however the strategy of exact combinations and harvesting times will depend on the final application. Soluble phenolic compounds with antioxidant activity would be optimized if 0DW sprouts are grown at 32°C and harvested between days 4 and 6. Phenolics with high antioxidant activity, regardless of the amount, would be optimized if non-wounded seeds are grown at 32°C and harvested at imbibition stage, if non-wounded seeds are grown at 18°C and harvested at day 2, or if 0DW seeds are grown at 25°C or 32°C and harvested at day 2. The optimum temperature yielding a non-wounded seed with high phenolic content and fresh appearance was 32°C, since 42°C caused seeds to spoil. However, a growing temperature of 25°C was chosen for the following chapters, since seeds exposed to 32°C grew too fast and complicated the experimental design and sampling process.

CHAPTER IV

POTENTIAL SIGNAL MOLECULES MEDIATING CHANGES IN PHENOLIC PROFILES IN MUNGBEAN SEEDLINGS IN RESPONSE TO GERMINATION, UV-C AND WOUNDING

Synopsis

From the different signal molecules (i.e. methyl jasmonate, ethylene, gibberellic acid and hydrogen peroxide) potentially mediating phenolic synthesis responses during germination, wounding and UV-C treatments, only exogenous hydrogen peroxide applications showed enhancement of phenylpropanoid synthesis in mungbean seeds, with enhancements similar to those of UV-C stress. Regarding identified individual phenolic compounds, there seemed to be enhanced synthesis of lignin precursors (i.e. *p*-coumaric, caffeic and ferulic acid esters) as well as synthesis of phytoalexins (i.e. flavanones and isoflavones) during germination and upon exposure to wounding and UV-C stresses. Wounding and UV-C stresses enhanced the amount of phenolic compounds synthesized when compared to non-treated controls; however, there was no evidence of synthesis of new phenolic compounds, indicating the possibility of a similar signal molecule mediating phenolic synthesis responses during germination, wounding and UV-C. Due to the referenced participation of reactive oxygen species (ROS) during lignification processes and phytoalexin synthesis, and the observed enhancement of phenylpropanoid synthesis on mungbean seeds with exogenous applications of hydrogen peroxide, we consider hydrogen peroxide to be a potential signal molecule mediating phenolic synthesis responses during germination, wounding and UV-C treatments. In addition, UV-C decreased fresh weight compared to controls and similar effect was obtained for exogenous hydrogen peroxide applications; therefore, we suggest a possible indole-3-acetic acid (IAA) inactivation mediated by UV-C elicited hydrogen peroxide.

Introduction

In the previous chapters (II and III) we determined that germination, UV-C and wounding enhanced the phenolic synthesis of mungbean seeds. At present it is not clear what are the critical signal molecules eliciting activation of phenylpropanoid enzymes in response to the above mentioned factors. Understanding the main role of phenolic synthesis and the type of synthesized phenolics in response to germination, UV-C and wounding, would allow determining if similar or different signal molecules mediate phenolic synthesis under these conditions. For example, stresses yielding different phenolic profiles will be most likely mediated by different signal molecules.

Phenolic compounds are secondary metabolites that are synthesized in plants mainly to protect them against harmful environmental conditions such as herbivore/pathogen attack, UV light stress, osmotic stress, hypoxia/hyperoxia stresses, among others (Taiz and Zieger 1998a, Blokhina and others 2003). However, no direct function has been attributed to phenolic compounds for normal growth and development functions such as photosynthesis, respiration, solute transport, translocation, nutrient assimilation, and differentiation (Taiz and Zieger 1998a). Functions of phenolic compounds during growth and development would be those of giving mechanical support to growing seedlings and to respond to signals of foreign attack that could compromise seed integrity. Mechanical support would be the most important, since lignin, a highly branched polymer of phenylpropanoid groups, strengthens stems and vascular tissue, thus allowing upward growth and permitting water and minerals to be conducted through the xylem under negative pressure without tissue collapse (Taiz and Zieger 1998a). In addition, lignin's physical toughness deters feeding by animals and its chemical durability makes it relatively indigestible to herbivores. Lignin is derived from the oxidative polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols; giving rise to the *p*-hydroxyphenyl, guaiacyl and syringyl units of lignin (Ascensao and Dubery 2003). Simple phenolic precursors for these compounds include *p*-coumaric acid, ferulic acid and sinapic acid (Davin and Lewis 1992). Other hydroxycinnamic acid esters have

been related to lignin cross-linking, mainly for strengthening the cell wall against potential damage (Ascensao and Dubery 2003).

Regarding UV-C, several studies have reported that UV-radiation, including UV-C, triggers phenylpropanoid metabolism in plants (Murphy and Huerta 1990, Arakawa 1988, Mercier and others 1994, Dong and others 1995, Jenkins and others 1997, Cantos and others 2001a, Reay and Lancaster 2001, Surjadinata 2006). Among the different UV types, UV-C is the most damaging to organisms due their short wavelengths with high energy (200-280 nm); however, the stratospheric ozone layer effectively blocks this UV radiation (Lumsden 1997, Hollosy 2002). Since plants have not evolved to respond to high levels of UV-C, it is very unlikely that they have specific receptors for these short wavelengths as is speculated for UV-B (280-320 nm) stresses, whose potential receptors could signal transduction processes, leading to the regulation of gene transcription (Jenkins and others 1997). With UV-B stresses, there is synthesis of UV-filtering compounds that absorb energy at UV-B wavelengths, such as flavonoids (λ_{max} at 270 and 345 nm) and hydroxycinnamic acids (λ_{max} at 320 nm) (Jenkins and others 1997). With respect to UV-C there is synthesis of phytoalexins (i.e. isocoumarins, isoflavonoids, stilbenes) and phenolic acids, thus mimicking the responses elicited by plant pathogens (Mercier and others 1993a, Mercier and others 1993b, Cantos and others 2001a, Cantos and others 2002, Chung and others 2003, Surjadinata 2006).

With wounding stress, physiological effects proportional to wounding intensity develop (Surjadinata 2006). When wounding stress occurs, the cell activates the specific transcriptional genes (Saltveit 2000, Leon and others 2001), with the purpose of adjusting the metabolism to repair and heal the damage and to synthesize compounds, such as lignin and suberin (Lagrimini 1991), to prevent invasion by predators. These processes could occur between a few minutes to several hours after wounding (Leon and others 2001). Wounding has been shown to increase respiration rate (Surjadinata and Cisneros-Zevallos 2003), ethylene production (Rolle and Chism 1987, Salveit 1997), moisture loss and membrane deterioration (Toivonen and DeEll 2002). Wounding responses could be generated either locally by the injured cells or systemically by the

adjacent cells (Leon and others 2001). Apart from the phenolic precursors for lignin, other phenolic compounds synthesized in response to wounding include hydroxycinnamic acids, hydroxybenzoic acids, coumarins and stilbenes (Facchini and others 2002, Rudolf and Resurreccion 2005, Surjadinata 2006).

Potential signal molecules for phenolic synthesis during germination, and wounding and UV-C stresses include gibberellic acid (GA), jasmonic acid (JA), ethylene and reactive oxygen species (ROS).

GA is a simple gibberellin, which promotes growth and elongation of cells (Taiz and Zeiger 1998c). Seed germination may require gibberellins for the activation of vegetative growth of the embryo, for weakening of a growth-constraining endosperm layer surrounding the embryo, and for mobilization of endosperm stored food reserves (Taiz and Zeiger 1998e). In grapevine pedicels, lignification and peroxidase seem to be controlled by GA (Ros Barcelo and others 2003). Since lignin is involved during vegetative growth and elongation, GA could be a signal molecule enhancing the synthesis of soluble phenolics as precursors for lignin biosynthesis during germination.

JA and methyl jasmonate (MJ), through the octadecanoid pathway, have been postulated to be one of the primary signals in the synthesis of plant secondary metabolites (Leon and others 2001, Zhao and others 2005). Endogenous and exogenous concentrations have shown to induce a wide variety of phenylpropanoid compounds (Zhao and others 2005). JA increases in different plants have been observed within minutes to several hours after wounding (Rakwal and Agrawal 2003) and usually, both JA and ethylene are simultaneously required for the activation of the wound signaling mechanism (Leon and others 2001).

Ethylene has shown to increase during plant growth and development processes including germination, senescence, cell elongation and fruit ripening, and during stress conditions, such as wounding, UV light, drought, salinity, flooding, and extreme temperatures (Kahl and Laties 1989, Ecker 1995, Lafuente and others 1996, Kieber 1997, Morgan and Drew 1997, Wang and others 2006). Wound-induced ethylene is involved in the initial stress responses such as abscission, senescence, wound healing,

and disease resistance, triggering synthesis of defense-related compounds, such as phenolics (Masia 2003). Exogenous ethylene applications in carrots have shown increases in phenolic compounds ranging from phenolic acids to isocoumarins (Fan and others 2000, Heredia 2006).

ROS could also be key signaling molecules for phenolic synthesis and are triggered by UV (including UV-C) and wounding stresses (Murphy and Huerta 1990, A-H-Mackerness and others 1999, A-H-Mackerness 2000, Stratman 2003, Liu and others 2005, Surjadinata 2006). ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) have been proposed as primary signals or messengers to elicit the synthesis of antioxidants and ROS scavenging enzymes during acclimatization, wounding, UV-C and other mild stresses (Murphy and Huerta 1990, Dat and others 2000, Purvis 2003). Superoxide and H_2O_2 have shown to induce different plant secondary metabolites such as furanocoumarins, isoflavonoids, saponins and *p*-coumaric acid derivatives (Zhao and others 2005).

Identifying potential signal molecules mediating phenolic biosynthesis would aid in developing strategies to control and re-direct phenolic synthesis in the desired direction. In addition, identifying the individual phenolics synthesized in response to dark germination, wounding and UV-C would allow a better understanding of possible relations between the stimulus, the signal molecules and the phenolic profiles synthesized for specific roles in the plant. In our mungbean seed model, several phenolic acid esters, flavonoids and isoflavones have been previously determined, being caffeoyltartronic and *p*-coumaroyltartronic acids the most abundant (Strack and others 1985, Snook and others 1993). Other compounds include robinin, rutin, kaempferol, quercetin, isoquercitrin, kaempferol-7-O-rhamnoside, nicotiflorin (kaempferol 3-rutinoside), vitexin, isovitexin, isovitexin-6”-O-rhamnoside, phaseollin, phaseollidin, dalbergioidin, kievitone, daidzein, daidzin, genistein, genistin, coumestrol, feruloyltartronic acid, cis *p*-coumaroyltartronic acid, cis caffeoyltartronic acid, delphinidin 3-glucoside, and traces of malvidin and pelargonidin glycosides. (Dewick and others 1970, Nozzolillo 1971, Strack and others 1985, Seneviratne and Harborne

1992, Larsen and others 1995, Kaufman and others 1997, Sawa and others 1999, Lal and others 2003).

In this chapter we determined the identity of some individual phenolics synthesized during germination and upon wounding and UV-C stresses, as well as the effect of different potential signal molecules on phenolic synthesis of mungbean seeds. Exogenous levels of potential signal molecules such as GA, MJ, ethylene and H_2O_2, were added for determining if their phenolic synthesis responses were similar to those of germination, UV-C and wounding. Signal molecule(s) eliciting phenolic synthesis responses similar to those of germination, wounding or UV-C were selected as the focus for further studies (Chapter V).

Materials and Methods

Materials

Mung bean (*Vigna radiata* L. Wilczek) seeds were purchased from Johnny's Selected Seeds (Winslow, ME, USA). Chlorogenic acid, MJ (95%), Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylene (CP grade, 99.5%), GA (99%) and H_2O_2 were obtained from Fisher Scientific (Houston, TX, USA).

Seed germination

Mungbean seeds were allowed to imbibe water for 14 h. Then water was removed and seeds were dark-germinated in glass jars with 3 layers of humidified paper towels. Paper towels were kept moist by spraying with sterile water as needed. Seeds were assayed for dry matter, total phenolics, total antiradical capacity (TAC) and HPLC phenolic profile identifications through time at the different experimental conditions.

Wounding, UV-C and exogenous application of potential signal molecules

For wounding treatment, mungbean seedlings were wounded at day 1 following water imbibition. Wounding was applied with a sterile razor blade and the seed was completely cut on both the longitudinal and latitudinal axis, to yield four sections. An incision was also applied parallel to the growing hypocotyl, without separating it from the attached seed section.

For UV-C, wounded and non-wounded mungbean seedlings grown for one day after imbibition were exposed to 240W UV-C for 40 min.

For ethylene treatments, whole seeds were exposed to continuous ethylene (0.27 to 1000 ppm) in sealed one gallon jars after imbibition. Controls were kept in sealed jars without exogenous ethylene. Continuous measurements of CO_2 were conducted for ensuring levels below 1%, thus avoiding potential toxic effects. Jars were ventilated twice daily and re-adjusted to the respective ethylene concentrations.

MJ (1000 uM, initially dissolved 3:1 in ethanol), GA (700 uM dissolved in 0.01M sodium phosphate) and H_2O_2 (400 mM) were applied to wounded seedlings at 1 day after water imbibition for 60 min. GA and H_2O_2 at the same levels were also applied to non-wounded seedlings. MJ (1 to 1000 uM, initially dissolved 3:1 in ethanol) was applied to whole seedlings at day 2 after water imbibition for 70 min.

Total phenolics and TAC were evaluated at days 0, 2, 4 and 6. Experiments were conducted at 18 °C, 22°C and 25 °C.

Total soluble phenolics

Total soluble phenolic content of methanolic extracts was assayed as described by Cevallos-Casals and Cisneros-Zevallos (2003) using Folin-Ciocalteu reagent. Measurements were conducted at 725 nm in a microtiter plate reader as described in Chapter III. Total phenolics were expressed as mg chlorogenic acid equivalents (CAE) 100 g^{-1} on a wet basis (WB), dry basis (DB), or per seed basis (PSB), based on a standard curve.

Total antiradical capacity (TAC)

TAC of phenolic compounds was adapted from Brand-Williams, Cuvelier, and Berset (1995) to be used in a microtiter plate reader as described in Chapter III. Thirteen μL of methanolic sample extract (equivalent volume of methanol for the blank) were mixed with 247 μL of DPPH solution (98.9 uM in methanol) inside each well of a 96-well flat bottom microtiter plate (Costar #3595, Corning, Inc., Corning, NY). Plates were tightly sealed with several layers of parafilm to prevent evaporation, placed in the dark at 20 °C and read at 515 nm after 20 h. The change in absorbance was used and results were expressed as ug Trolox equivalents g^{-1} on a WB or DB, from a standard curve.

High performance liquid chromatography (HPLC) identification of phenolic compounds

HPLC was performed by separating phenolic compounds on an Atlantis C18 column (5 μm, 4.6 mm x 150 mm) using a binary Waters 515 HPLC pump system, a Waters 717 plus autosampler automated gradient controller and a Waters 996 Photodiode array detector. The mobile phase was composed of solvent A (pH 2.3 nanopure water) and solvent B (acetonitrile). Elution was as follows: isocratic conditions from 0-5 min with 85% A and 15% B. Gradient conditions from minute 5 to 30 starting with 85% A (15% B) and ending with 0% A (100% B). Then, isocratic conditions from minute 30 to minute 35 with 0% A and 100% B. The flow rate was 1 mL/min and 10 μL of sample were injected. The temperature of the column was kept at 35 ºC. Phenolic compounds were identified by comparing retention time and spectra to those of external standards as well as to those of published references. Alkaline or alkaline + acid hydrolyses of the samples were performed for facilitating compound identification.

Alkaline hydrolysis was conducted following the protocol by Llorach and others (2003) with some modifications. Hydrolysis was performed by adding 0.5 mL 6N NaOH to 1 mL of methanolic sample extract and incubating the mixture for 2 h in a

screw capped polypropylene tube under a nitrogen atmosphere. For alkaline + acid hydrolysis, concentrated HCl was added to previously saponified samples so as to reach pH ~1, after which, samples were incubated at 75°C for 30 min. Samples subject to alkaline and alkaline + acid hydrolysis were injected for HPLC identification. Alkaline hydrolysis serves to release acyl groups from the phenolic compounds if present and adding a mild acid hydrolysis helps release any potentially attached sugars.

Samples that were injected for phenolic identification included imbibed mungbean seeds (6 seeds homogenized with 20 mL methanol), 6 d old seeds grown at 18°C (6 seeds homogenized with 20 mL methanol), 4 d old seeds grown at 25°C (2.3 g homogenized with 20 mL methanol), 4 d old seeds grown at 25°C and wounded after imbibition (2.3 g homogenized with 20 mL methanol), and 4 d old seeds grown at 25°C and UV-C irradiated 1 d after imbibition (2.3 g homogenized with 20 mL methanol). Samples were centrifuged at 29000g, the supernatant filtered through a 0.2 um syringe filter and subsequently injected into the HPLC system. Injections were made in duplicate, and each injection came from two independent replicates, completing a total of 4 replicates per sample.

Analysis of variance

One-way analysis of variance (ANOVA) was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Means were compared with Duncan's Multiple Range Test at $\alpha = 0.05$.

Results and Discussion

Potential signal molecules mediating synthesis of phenolic antioxidant compounds during germination and upon exposure to UV-C and wounding stresses

Effect of MJ and ethylene on phenolic synthesis of non-wounded mungbean seeds

In dose response studies, neither MJ nor ethylene were able to elicit phenolic synthesis responses similar to those previously observed for UV-C and wounding in Chapters II and III (Figures 19, 20). MJ at lower levels (1 to 100 uM) had significantly

($\alpha = 0.05$) lower phenolic levels on a DB than control. With ethylene, there was an inverse relation between seed growth and ethylene concentration. Work on etiolated pea seedlings has shown ethylene to change the growth pattern of seedlings by reducing the rate of elongation and increasing lateral expansion, leading to swelling of the region below the hook (Taiz and Zieger 1998f). The effect of decreasing seed growth by exogenous ethylene caused phenolic yield per seed to decrease with increasing ethylene concentrations (Figure 20A).

These results suggest that neither of these two plant hormones seems to mediate phenolic synthesis during germination, UV-C stress and wounding. Work by Heredia (2006), showed that exogenous applications of ethylene and methyl jasmonate hormones on different whole plant tissues did not induce significant phenolic synthesis on different fruits, vegetables, roots and tubers. Regarding UV-C stress, lack of ethylene induction was observed in irradiated zucchini squash (Erkan and others 2001).

Effect of GA and H_2O_2 on phenolic synthesis of non-wounded mungbean seeds at different germination stages

Immediately after water imbibition, H_2O_2 affected seeds by slightly decreasing their phenolic contents compared to seeds imbibed in pure water (Figure 21A, C). It is possible that phenolics in the seed coat could have been oxidized, as this has been determined to be a mechanism for promoting germination, since monomeric phenolics are considered germination inhibitors (Ogawa and Iwabuchi 2001). At other germination stages, GA affected seeds by lowering their phenolic synthesis with respect to the control, while H_2O_2 elicited increases similar to those of UV-C, especially at day 2 after water imbibition (Figure 21A, C). Seeds exposed to UV-C or to hydrogen peroxide had lower fresh weights compared to controls and gibberellic acid treated seeds (Figure 22). It is possible that both, hydrogen peroxide and UV-C affected directly or indirectly the plant growth hormone regulator indole-3-acetic acid (IAA). In general, these results show GA to be an inhibitor of phenolic synthesis, while H_2O_2 to be a potential signal molecule mediating phenolic synthesis during germination and UV-C. The similar

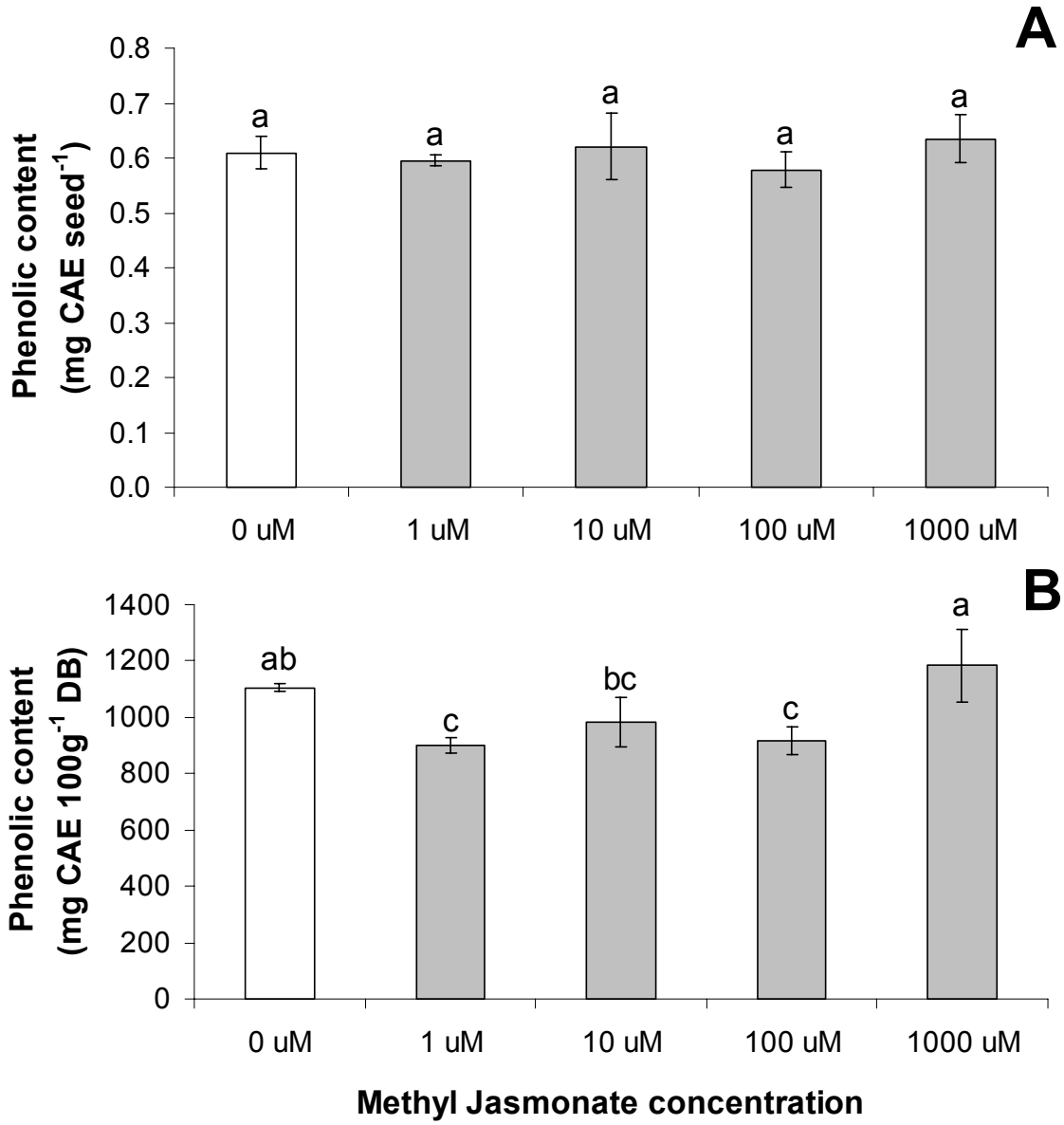

Figure 19 – Effect of increasing methyl jasmonate concentrations on phenolic content of mungbean seedlings grown for 6 d at 18°C. A: phenolic content per seed basis (PSB). B: phenolic content dry basis (DB). Columns within each figure with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Two day old seedlings were dipped in methyl jasmonate solutions for 70 min. Bars show the average of 3 replicates ± standard deviation.

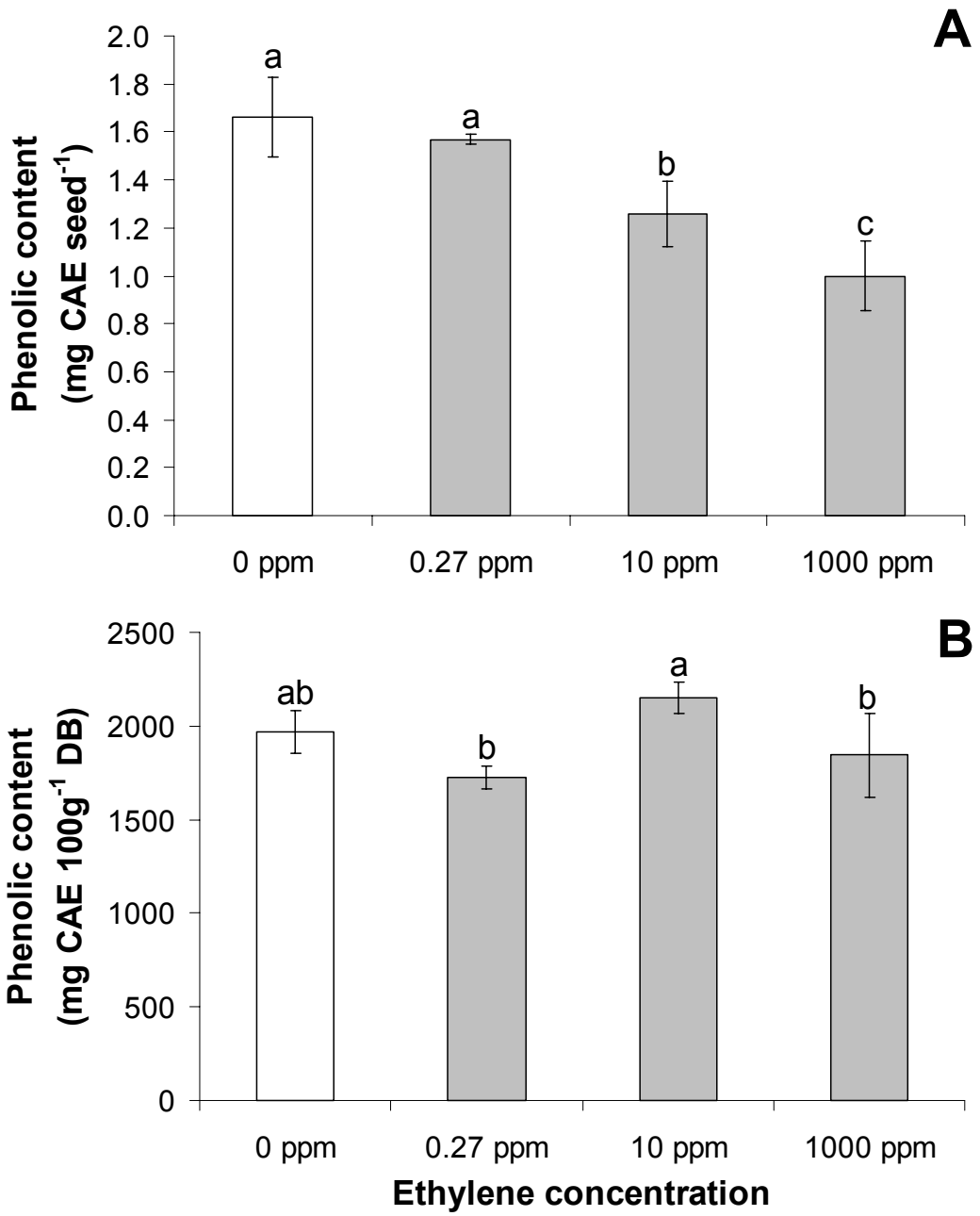

Figure 20 – Effect of increasing ethylene concentrations on phenolic content of mungbean seedlings grown for 6 d at 22°C. A: phenolic content per seed basis (PSB). B: phenolic content dry basis (DB). Columns within each figure with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Imbibed seeds were continuously exposed to ethylene during germination. Bars show the average of 3 replicates ± standard deviation.

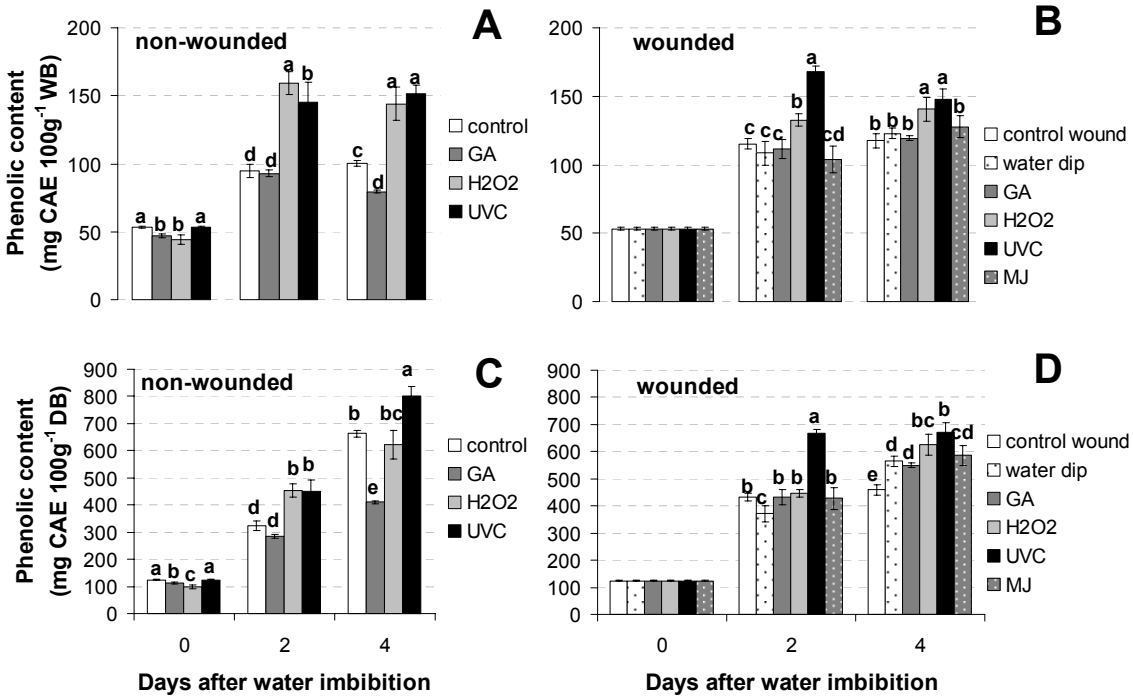

Figure 21 – Comparison on the effect of gibberellic acid, hydrogen peroxide and methyl jasmonate on soluble phenolic contents of non-wounded and wounded mungbean seeds grown at 25°C to those of germination, wounding and UV-C treatments without these chemicals. A, B: phenolic content on a wet basis (WB). C, D: phenolic content on a dry basis (DB). A, C: non-wounded seeds. B, D: wounded seeds. Columns within the same germination stage and within the same reporting units with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Wounding and UV-C treatments were applied at day 1 after imbibition. For chemical treatments, 1 d old seeds were dipped in gibberellic acid (700 uM), hydrogen peroxide (400 mM) or methyl jasmonate (1000 uM) for 1 h. Bars show the average of 3 replicates ± standard deviation.

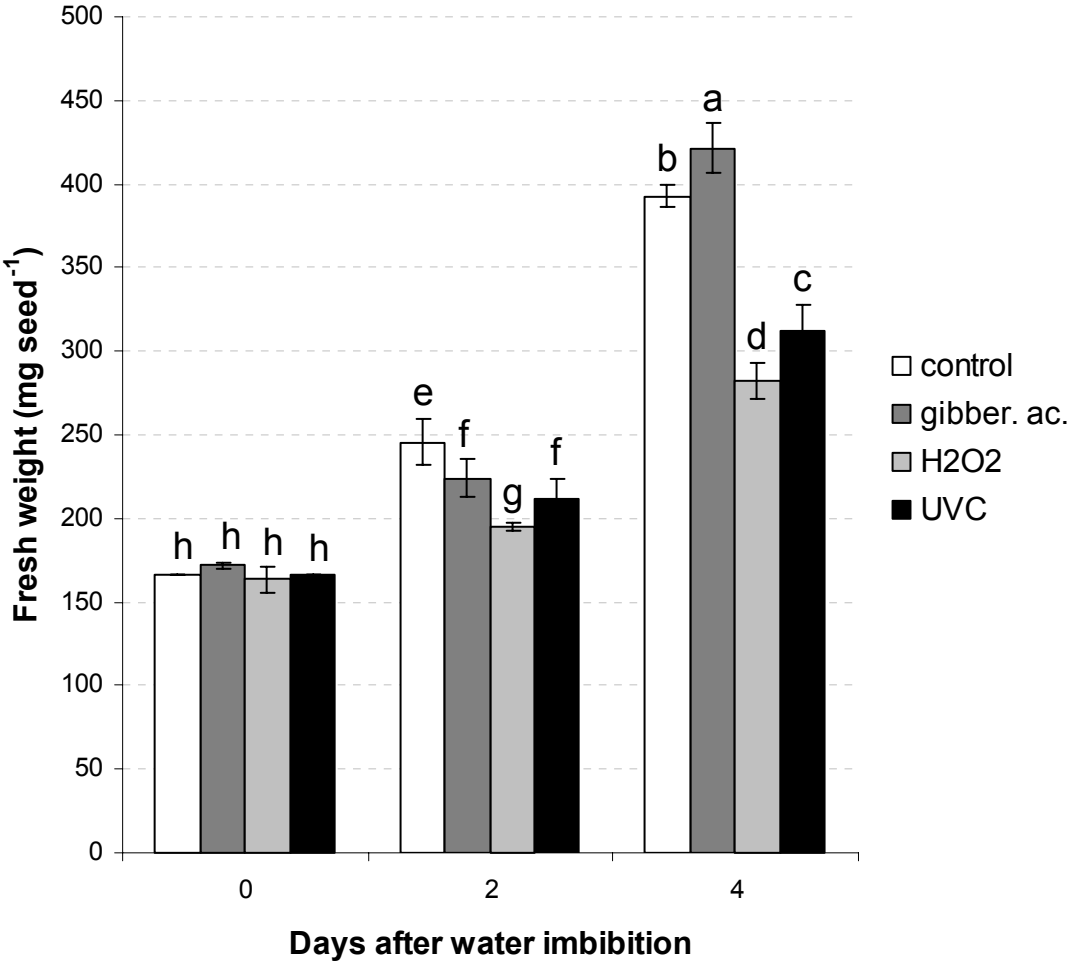

Figure 22 – Comparison on the effect of gibberellic acid and hydrogen peroxide on fresh weight of non-wounded mungbean seeds grown at 25°C to those of UV-C treated seeds. Columns with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. UV-C treatment was applied at day 1 after imbibition. For chemical treatments, 1 d old seeds were dipped in gibberellic acid (700 uM) or hydrogen peroxide (400 mM) for 1 h. Bars show the average of 3 replicates ± standard deviation

effects on decreased fresh weights caused by hydrogen peroxide and UV-C stress could indicate an increase in hydrogen peroxide during UV-C stress as well as its probable mediation of IAA inactivation.

Effect of MJ, GA and H_2O_2 on phenolic synthesis of wounded mungbean seeds at different germination stages

Different chemicals were applied to wounded areas for determining their role as phenolic synthesis mediators on these exposed tissues. Wounded seeds dipped in pure water instead of chemical solution, served as controls, since there could be leaching of important compounds, including phenolics and enzymes. Water-dipped controls had lower phenolic content on a DB at day 2 when compared to non-dipped control, but higher phenolic content at day 4, which could be explained by a leaching of soluble phenolic compounds and enzymes that transform or degrade synthesized phenolics. All chemical treatments at day 2 after water imbibition yielded higher phenolic contents on a DB than water-dipped controls and similar contents than non-dipped controls (Figure 21C). However, at day 4 after water imbibition only H_2O_2 treatment was significantly ($\alpha = 0.05$) different than both controls. It was observed that the response of H_2O_2 at day 4 on wounded tissue was very similar to that of UV-C on wounded tissue. These results suggest that H_2O_2 can be considered a potential mediator of wounding and UV-C stresses. With respect to exogenous applications of methyl jasmonate, work by Heredia (2006) on 16 different wounded fruits, vegetables, roots and tubers, showed significant increases only in celery, lettuce and carrot, indicating a tissue-specific selectivity of methyl jasmonate mediated phenylpropanoid induction.

Regarding the synthesis of phenolics due to UV-C on wounded tissues, we observed a synergistic effect when combining both UV-C and wounding stresses (Figure 21C, D). Phenolic increases on whole seeds due to UV-C alone at day 2 after imbibition were ~38.5% and due to wounding stress alone ~33.0%. Additive responses would have yielded an increase of ~71.5%; however an increase of ~105.5% was observed when combining UV-C and wounding stresses, thus showing a synergistic effect. This

synergism between wounding and UV-C has also been observed on carrot tissues (Surjadinata 2006). We hypothesize that this synergism occurs since both stresses could be mediated by the same signal molecule. Unified responses towards the synthesis of a specific target signal molecule would yield a synergistic response. Wounding and UV-C stress could stimulate the accumulation of ROS, through activation of membrane-bound NADPH-oxidase (Dat and others 2000, Surjadinata 2006) and possibly through water ionization. H_2O_2 is speculated to be an important ROS mediating stress-induced responses of wounding and UV-C stresses (Dat and others 2000).

Regarding the specific synthesis of phenolic antioxidants, TAC results were similar to those of total soluble phenolics, confirming similar synthesis effects of exogenous hydrogen peroxide applications to those of UV-C treatment (Figure 23). When wounding and UV-C stresses were combined, antagonistic responses were observed for TAC, as compared to synergistic effects for soluble phenolics. TAC increases on whole seeds due to UV-C alone at day 2 after imbibition were ~60.8% and due to wounding stress alone ~22.3% (Figure 23C, D). Additive responses would have yielded an increase of ~83.1%; however an increase of ~73.7% was observed when combining UV-C and wounding stresses, thus showing an antagonistic effect. These differences in response between soluble phenolics and TAC are most likely due to enhanced synthesis of phenolic antioxidants with low antioxidant activity or transformation/degradation of phenolics with high antioxidant properties with combination of wounding and UV-C stresses.

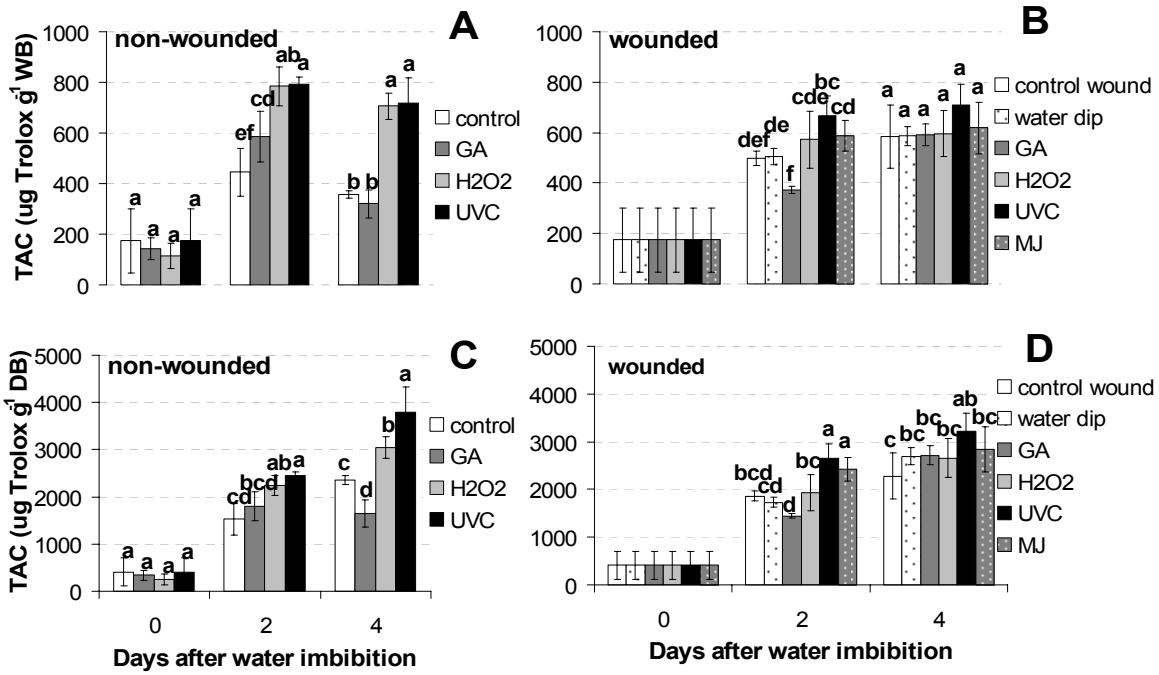

Figure 23 – Comparison on the effect of gibberellic acid, hydrogen peroxide and methyl jasmonate on antioxidant activity of wounded and non-wounded mungbean seeds grown at 25°C to those of germination, wounding and UV-C treatments without these chemicals. A, B: antioxidant activity on a wet basis (WB). C, D: antioxidant activity on a dry basis (DB). A, C: non-wounded seeds. B, D: wounded seeds. Columns within the same germination stage and within the same reporting units with similar letters are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Wounding and UV-C treatments were applied at day 1 after imbibition. For chemical treatments, 1 d old seeds were dipped in gibberellic acid (700 uM), hydrogen peroxide (400 mM) or methyl jasmonate (1000 uM) for 1 h. Bars show the average of 3 replicates ± standard deviation.

HPLC profiles of phenolic compounds synthesized during germination and after exposure to UV-C and wounding stresses

Phenolic synthesis during dark germination

Water imbibed seeds showed the presence of three major phenolic compounds, the flavone glycosides, isovitexin (33.8%) and vitexin (50.8%); and a trihydroxyflavanone (3.1%) (Figures 24, Table 2). An aromatic amino acid (12.3%) was also detected in these imbibed seeds (Figures 24, Table 2). During germination at 18°C for 6 d, 8 new phenolic compounds were synthesized in mungbean seedlings (Figures 24, Table 2). Compounds present in these 6 d old seeds included tartronic acid esters (38%), flavones (28.4%), an aromatic amino acid (21.4%), flavanones (7.4%), coumestans (~3.1%), a flavonol (1.7%), and a hydroxycinnamic acid (1.3%).

Seeds germinated at 25°C for 4 d, showed lower contents of vitexin and isovitexin when compared to those germinated at 18°C for 6 d; however, higher amounts of caffeoyltartronic acid, flavanones, and the presence of 5 new peaks was observed (Figure 25, Table 2). These results indicate that higher temperatures seem to speed up the transformation of vitexin and isovitexin and enhance phenylpropanoid metabolism in general, as was observed in Chapter III. Alkaline hydrolysis was conducted for confirming the identity of unknown compounds, especially the predominant tartronic acid esters. After hydrolysis, results showed the disappearance of the tartronic acid esters (peaks 4, 5 and 6) and the appearance of the released hydroxycinnamic acids (peaks 19, 20, 21 and 22) (Figure 25, Table 2). The tartronic acid esters were presumed to be caffeoyltartronic acid (peak 4), *p*-coumaroyltartronic acid (peak 5) and feruloyltartronic acid (peak 6), since after hydrolysis appeared a hydroxycinnamic acid (peak 21), *p*-coumaric acid (peak 19), ferulic acid (peak 20) and a coumaric acid derivative (peak 22). Peak assignments for these tartronic acid esters were complemented based on their order of elution and absorption maxima in the UV range (λmax) when compared to published results by Strack and others (1985). These authors have also determined the mentioned tartronic acid esters to be the main phenolic compounds present in mungbean seedlings (Strack and others 1985). Adding a mild

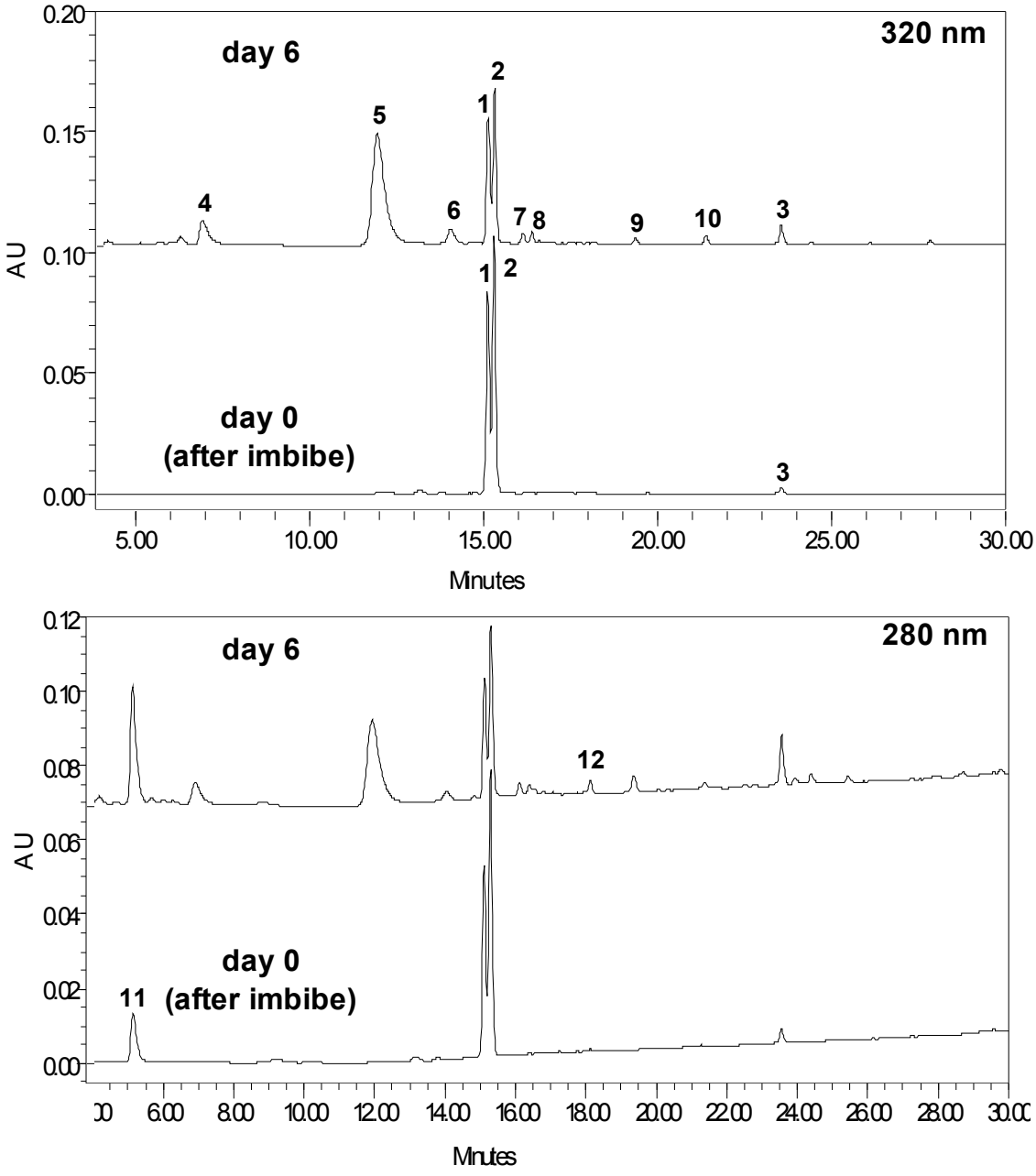

Figure 24 – HPLC profiles of imbibed and 6 d old mungbean seeds grown at 18°C. Peaks 1 = vitexin, 2 = isovitexin, 3 = trihydroxyflavanone, 4 = caffeoyltartronic acid, 5 = *p*-coumaroyltartronic acid, 6 = feruloyltartronic acid, 7 = kaempferol 3-rutinoside, 8 = flavanone, 9 = coumestan, 10 = coumestan, 11 = aromatic amino acid, 12 = hydroxycinnamic acid derivative. AU = absorbance units. X-axis is retention time in min.

Table 2 – Identity, relative abundance and characteristics of phenolic compounds and hydrolyzed phenolic compounds present on water imbibed and germinated mungbean seeds.

	Peak #	Compound	Retention time (min)	λ max (nm)	Relative area 320 nm (%)	Relative area 280 nm (%)
control after imbibe	1	vitexin	15.1	213; 267.3; 336.2	43.0	33.8
	2	isovitexin	15.3	211.8; 268.4; 336.2	55.6	50.8
	3	trihydroxyflavanone	23.6	194.2; 294.5	1.4	3.1
	11	aromatic amino acid	5.2	217.7; 277.9	0.0	12.3
control at day 6, 18°C	1	vitexin	15.1	213; 267.3; 336.2	14.0	11.3
	2	isovitexin	15.3	211.8; 268.4; 336.2	17.4	17.1
	3	trihydroxyflavanone	23.6	194.2; 294.5	2.5	5.2
	4	caffeoyltartronic acid	6.9	195.4; 327.9	6.6	5.2
	5	*p*-coumaroyltartronic acid	11.9	191.9; 227.1; 313.6	49.2	30.6
	6	feruloyltartronic acid	14.0	194.2; 216.5; 240.1; 326.7	4.0	2.2
	7	Kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	1.2	1.7
	8	flavanone	16.4	194.2; 267.3; 336.2	1.5	2.2
	9	coumestan	19.4	195.4; 283.8; 338.6	0.7	1.9
	10	coumestan	21.4	204.8; 243.6; 342.2	1.2	0.0
	11	aromatic amino acid	5.1	217.7; 277.9	0.0	21.4
	12	hydroxycinnamic acid derivative	18.1	194.2; 275.5	0.0	1.3
control at day 4, 25°C no hydrolysis	1	vitexin	15.0	213; 267.3; 336.2	0.8	1.4
	2	isovitexin	15.2	211.8; 268.4; 336.2	2.7	2.1
	3	trihydroxyflavanone	23.6	194.2; 294.5	6.7	10.6
	4	caffeoyltartronic acid	6.5	195.4; 327.9	23.7	10.2
	5	*p*-coumaroyltartronic acid	11.0	191.9; 227.1; 313.6	42.5	20.3
	6	feruloyltartronic acid	13.3	194.2; 216.5; 240.1; 326.7	2.6	0.0
	7	kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	3.1	2.1
	8	flavanone	16.3	194.2; 267.3; 336.2	1.9	0.0
	9	coumestan	19.3	195.4; 283.8; 338.6	0.7	1.5
	10	coumestan	21.4	204.8; 243.6; 342.2	1.0	1.4
	11	aromatic amino acid	5.2	217.7; 277.9	0	20.5
	12	hydroxycinnamic acid derivative	18.1	195.4; 281.5	0.0	1.8
	13	hydroxycinnamic acid derivative	14.7	216.5; 325.5	0.8	0.0
	14	quercetin 3-glycoside	15.1	202.4; 254.2; 355.4	1.7	1.5
	15	co-elution	20.0	193.1; 220.1; 293.3	9.0	21.0
	16	ester-bound phenolic	22.7	208.3; 240.1; 319.5; 335	1.0	0.0
	17	trihydroxyflavanone	24.4	195.4; 289.8	1.0	3.5
alkaline hydrolysis	1	vitexin	15.0	213; 267.3; 336.2	2.8	1.7
	2	isovitexin	15.3	211.8; 268.4; 336.2	2.6	1.7
	3	trihydroxyflavanone	23.6	194.2; 294.5	3.4	3.5
	7	kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	2.9	2.0
	10	coumestan	21.5	204.8; 243.6; 342.2	2.1	1.9
	11	aromatic amino acid	5.2	217.7; 277.9	0.0	13.3
	14	quercetin 3-glycoside	15.1	204.8; 255.4; 354.2	1.3	0.0
	15	co-elution	20.2	193.1; 220.1; 293.3	14.8	25.5
	17	trihydroxyflavanone	24.3	195.4; 289.8	1.2	1.7
	18	quercetin glycoside	8.6	191.9; 215.4; 296.9; 319.5	4.0	2.3
	19	*p*-coumaric acid	14.5	224.8; 308.8	34.6	20.9
	20	ferulic acid	15.6	191.9; 215.4; 238.9; 324.3	5.0	2.8
	21	hydroxycinnamic acid derivative	18.1	193.1; 216.5; 240.1; 325.5	9.6	4.1
	22	coumaric acid derivative	20.6	193.1; 209.5; 225.9; 310	10.3	6.7
alkaline + acid hydrolysis	1	vitexin	15.1	213; 267.3; 336.2	3.5	2.1
	2	isovitexin	15.3	211.8; 268.4; 336.2	3.3	1.9
	3	trihydroxyflavanone	23.6	194.2; 294.5	2.7	2.6
	7	kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	3.3	2.2
	10	coumestan	21.5	204.8; 243.6; 342.2	2.0	1.1
	11	aromatic amino acid	5.2	217.7; 277.9	0.0	15.6
	14	quercetin 3-glycoside	15.2	204.8; 255.4; 354.2	1.4	0.0
	15	co-elution	20.0	193.1; 220.1; 293.3	8.5	14.1
	17	trihydroxyflavanone	24.3	195.4; 289.8	1.3	0.0
	18	quercetin glycoside	8.4	191.9; 215.4; 296.9; 319.5	2.3	1.8
	19	*p*-coumaric acid	14.5	224.8; 308.8	40.2	23.2
	20	ferulic acid	15.6	191.9; 215.4; 238.9; 324.3	5.6	3.0
	21	hydroxycinnamic acid derivative	18.1	193.1; 216.5; 240.1; 325.5	6.2	2.4
	22	coumaric acid derivative	20.6	193.1; 209.5; 225.9; 310	11.1	7.3

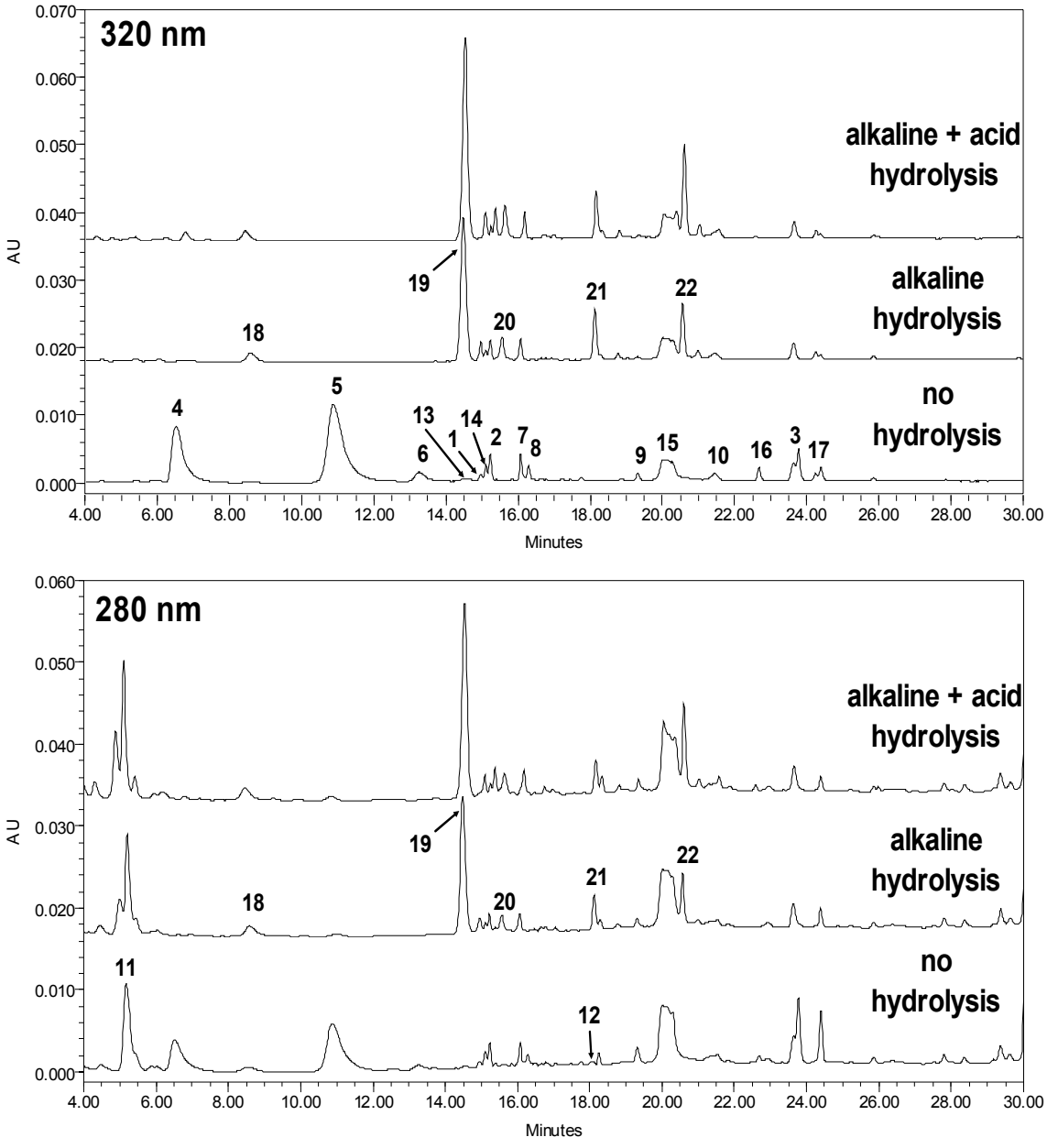

Figure 25 – HPLC profiles of 4 d old mungbean seeds grown at 25°C and subject to alkaline and alkaline + acid hydrolyses. Peaks 1 = vitexin, 2 = isovitexin, 3 = trihydroxyflavanone, 4 = caffeoyltartronic acid, 5 = *p*-coumaroyltartronic acid, 6 = feruloyltartronic acid, 7 = kaempferol 3-rutinoside, 8 = flavanone, 9 = coumestan, 10 = coumestan, 11 = aromatic amino acid, 12 = hydroxycinnamic acid derivative, 13 = hydroxycinnamic acid derivative, 14 = quercetin 3-glycoside, 15 = co-elution, 16 = ester-bound phenolic, 17 = trihydroxyflavanone, 18 = quercetin glycoside, 19 = p-coumaric acid, 20 = ferulic acid, 21 = hydroxycinnamic acid derivative, 22 = coumaric acid derivative. AU = absorbance units. X-axis is retention time in min.

acid hydrolysis to the previously saponified samples slightly decreased the content of glycosides and increased the amounts of some hydroxycinnamic acids; however no new compounds were released (Figure 25, Table 2).

The high amount of vitexin and isovitexin at initial germination stages of mungbean seeds has previously been determined, especially concentrating in seed coat and cotyledon sections, together with their decrease with increasing germination and the total disappearance of vitexin beyond day 6 of germination (Larsen and others 1995). Regarding their structures, vitexin and isovitexin have 7 and 6 hydroxyl groups, respectively; feature that could make them very efficient antioxidants. The high level of phenolic specific antioxidant activity for mungbean seeds observed in Chapters II and III during early germination stages could be due to the high content of these antioxidant compounds. Previous work has shown that vitexin scavenges intracellular superoxide radicals in cultured human dermal fibroblasts (Kim and others 2005). Antioxidant activity in situ of plant flavones has not been demonstrated; however, it is possible that the role of these compounds during early germination stages is to scavenge excess reactive oxygen species (ROS), due to a possible oxidative burst during germination. Apart from potentially acting as antioxidants in situ, it is possible that these flavones could act as antimicrobials as well. Antimicrobial activity for vitexin has previously been determined (Salvador and others 2004) and its induction, together with isovitexin, has been observed in cucumber leaves upon infection against powdery mildew fungi (McNally and others 2003).

Phenolic synthesis due to wounding and UV-C stresses

When mungbean seeds were exposed to wounding and UV-C stresses, some phenolic compounds increased while others decrease, when compared to 4 d old mungbean controls; however, an overall increase in compound peak areas at 280 nm was observed when compared to controls (Figure 26, Table 3). Increases in total 280 nm peak compound areas for wounding and UV-C treatments compared to controls were

11% and 41%, respectively; while increases in total 320 nm areas were less evident (-3% and 3% for wounding and UV-C treatments, respectively).

Compounds decreasing due to wounding were isovitexin, all tartronic acid esters, all flavonols and other phenolic acids and esters. Compounds increasing were all phytoalexins, including vitexin, several flavanones and all coumestans (Figure 26, Table 3). Two new flavanones were quantified due to wounding stress (peaks 1' and 2') which seemed to be present in controls but at very low levels to allow detection and quantification (Figure 26). Regarding decreases in tartronic acid esters, previous studies have shown that caffeoyltartronic acid levels decrease in mungbean seedlings when primary leaves are detached (Strack and others 1985), thus triggering a wounding response.

For UV-C treatment, compounds decreasing included isovitexin and all tartronic acid esters; while those increasing were vitexin, all flavonols, all flavanones, all coumestans and other phenolic acids and esters (Figure 26, Table 3). In addition to increases in flavanone peaks 1' and 2' (observed also with wounding stress), two other flavanones and two isoflavones appeared (peaks 3', 4', 5' and 6'). Peaks 1' through 6' seem to be present in controls and wounded seeds; however, due to their low presence, quantification and identification were not possible.

All phenolic compounds synthesized in mungbean seeds during germination, wounding and UV-C treatments are related to each other since all are derived from phenylalanine and some serve as precursors for others; however, their synthesis depends on activation of specific key enzymes (Figure 27). For example, *p*-coumaric acid, an important phenolic, could be transformed into lignin either by previous conversions to caffeic and ferulic acids, or through *p*-coumaroyl-CoA. This last compound serves as precursor for chalcones, which later get transformed into flavanones. Flavanones can be either transformed into flavonols, flavones or isoflavones. Within isoflavones, the observed coumestans are derived via cyclization from daidzein (Durango and others 2002).

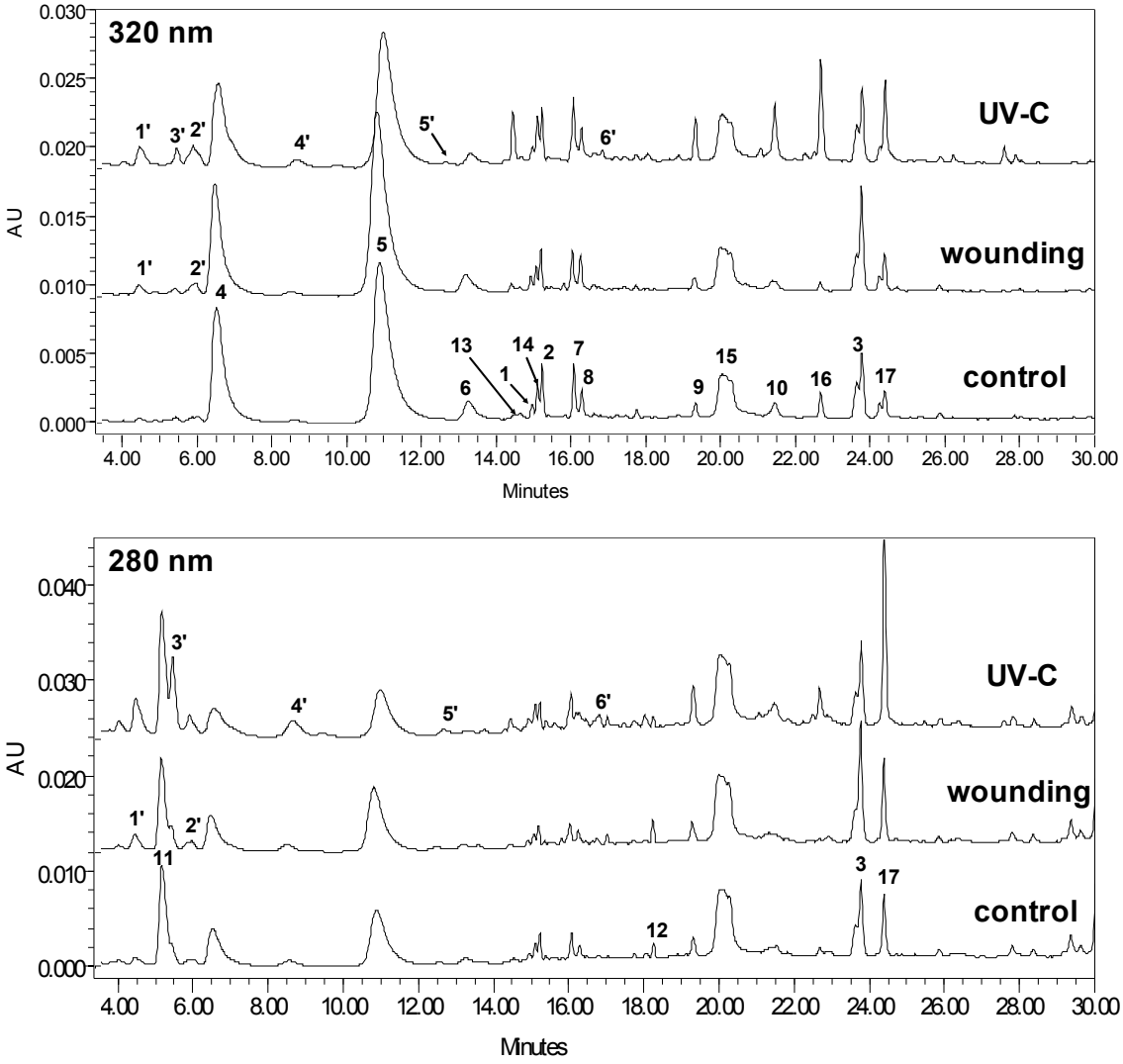

Figure 26 – HPLC profiles of 4 d old mungbean seeds grown at 25°C without stress or subject to wounding or UV-C stresses. Peaks 1 = vitexin, 2 = isovitexin, 3 = trihydroxyflavanone, 4 = caffeoyltartronic acid, 5 = *p*-coumaroyltartronic acid, 6 = feruloyltartronic acid, 7 = kaempferol 3-rutinoside, 8 = flavanone, 9 = coumestan, 10 = coumestan, 11 = aromatic amino acid, 12 = hydroxycinnamic acid derivative, 13 = hydroxycinnamic acid derivative, 14 = quercetin 3-glycoside, 15 = co-elution, 16 = ester-bound phenolic, 17 = trihydroxyflavanone, 1' = trihydroxyflavanone, 2' = flavanone, 3' = trihydroxyflavanone, 4' = trihydroxyflavanone, 5' = daidzin, 6' = isoflavone. AU = absorbance units. X-axis is retention time in min.

Table 3 – Identity and relative abundance of phenolic compounds present in 4 d old wounded and UV-C irradiated mungbean seeds compared to controls.

	Peak #	Compound	Ret. time (min)	λ max (nm)	Relative area 320 nm (%)	% change at 320 nm from control	Relative area 280 nm (%)	% change at 280 nm from control
wounding	1	vitexin	14.9	213; 267.3; 336.2	0.8	2.9	0.0	ND*
	2	isovitexin	15.2	211.8; 268.4; 336.2	2.0	-25.9	1.2	-34.9
	3	trihydroxyflavanone	23.6	194.2; 294.5	8.7	25.9	16.4	74.2
	4	caffeoyltartronic acid	6.5	195.4; 327.9	18.5	-24.0	6.6	-27.5
	5	*p*-coumaroyltartronic acid	10.8	191.9; 227.1; 313.6	43.5	-0.2	17.5	-3.1
	6	feruloyltartronic acid	13.2	194.2; 216.5; 240.1; 326.7	2.0	-24.2	0.0	ND*
	7	kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	2.6	-19.1	2.0	6.8
	8	flavanone	16.3	194.2; 267.3; 336.2	1.8	-5.8	1.4	ND**
	9	coumestan	19.3	195.4; 283.8; 338.6	0.8	9.2	1.6	17.2
	10	coumestan	21.4	204.8; 243.6; 342.2	1.1	9.7	1.1	20.4
	11	aromatic amino acid	5.2	217.7; 277.9	0.0	ND*	14.1	-22.6
	12	hydroxycinnamic acid derivative	18.2	195.4; 281.5	0.0	ND*	1.2	134.3
	13	hydroxycinnamic acid derivative	14.4	216.5; 325.5	0.7	-8.4	0.0	ND*
	14	quercetin 3-glycoside	15.1	202.4; 254.2; 355.4	1.3	-26.8	0.9	-28.5
	15	co-elution	20.0	193.1; 220.1; 293.3	9.2	-0.4	20.0	7.1
	16	ester-bound phenolic	22.7	208.3; 240.1; 319.5; 335	0.7	-34.6	0.0	ND*
	17	trihydroxyflavanone	24.3	195.4; 289.8	3.0	179.3	6.6	113.1
	1'	trihydroxyflavanone	4.5	198.9; 255.4; 320.7	1.2	ND**	2.8	ND**
	2'	flavanone	5.9	193.1; 257.8; 320.7	1.0	ND**	1.6	ND**
UV-C	1	vitexin	15.0	213; 267.3; 336.2	0.7	3.4	1.2	24.8
	2	isovitexin	15.2	211.8; 268.4; 336.2	2.4	-4.6	1.4	-5.5
	3	trihydroxyflavanone	23.6	194.2; 294.5	6.8	8.9	7.8	7.8
	4	caffeoyltartronic acid	6.6	195.4; 327.9	16.9	-22.6	4.5	-35.1
	5	*p*-coumaroyltartronic acid	11.0	191.9; 227.1; 313.6	31.7	-19.3	10.6	-23.8
	6	feruloyltartronic acid	13.3	194.2; 216.5; 240.1; 326.7	1.8	-22.1	0.0	ND*
	7	kaempferol 3-rutinoside	16.1	194.2; 263.7; 348.2	3.3	14.3	2.2	46.2
	8	flavanone	16.3	194.2; 267.3; 336.2	2.0	17.0	2.5	ND**
	9	coumestan	19.3	195.4; 283.8; 338.6	1.7	156.5	2.0	87.1
	10	coumestan	21.5	204.8; 243.6; 342.2	2.5	175.9	1.9	162.0
	11	aromatic amino acid	5.2	217.7; 277.9	0.0	ND*	13.3	-5.5
	13	hydroxycinnamic acid derivative	14.4	216.5; 325.5	2.2	195.5	1.2	ND**
	14	quercetin 3-glycoside	15.1	202.4; 254.2; 355.4	1.9	18.2	1.3	29.2
	15	co-elution	20.0	193.1; 220.1; 293.3	8.3	0.3	17.2	1.9
	16	ester-bound phenolic	22.7	208.3; 240.1; 319.5; 335	5.0	441.7	2.3	ND**
	17	trihydroxyflavanone	24.3	217.7; 277.9	4.4	-20.8	8.3	246.8
	1'	trihydroxyflavanone	4.5	198.9; 255.4; 320.7	1.9	ND**	4.1	ND**
	2'	flavanone	5.9	193.1; 257.8; 320.7	2.4	ND**	2.0	ND**
	3'	trihydroxyflavanone	5.5	197.8; 282.7	1.0	ND**	6.2	ND**
	4'	trihydroxyflavanone	8.7	194.2; 289.8	0.0	ND*	2.2	ND**
	5'	daidzin	12.7	191.9; 247.2	0.0	ND*	2.1	ND**
	6'	isoflavone	16.8	194.2; 229.5; 281.5	0.0	ND*	1.5	ND**

ND* = not detected in wounding or UV-C treatments, ND** = not detected in controls.

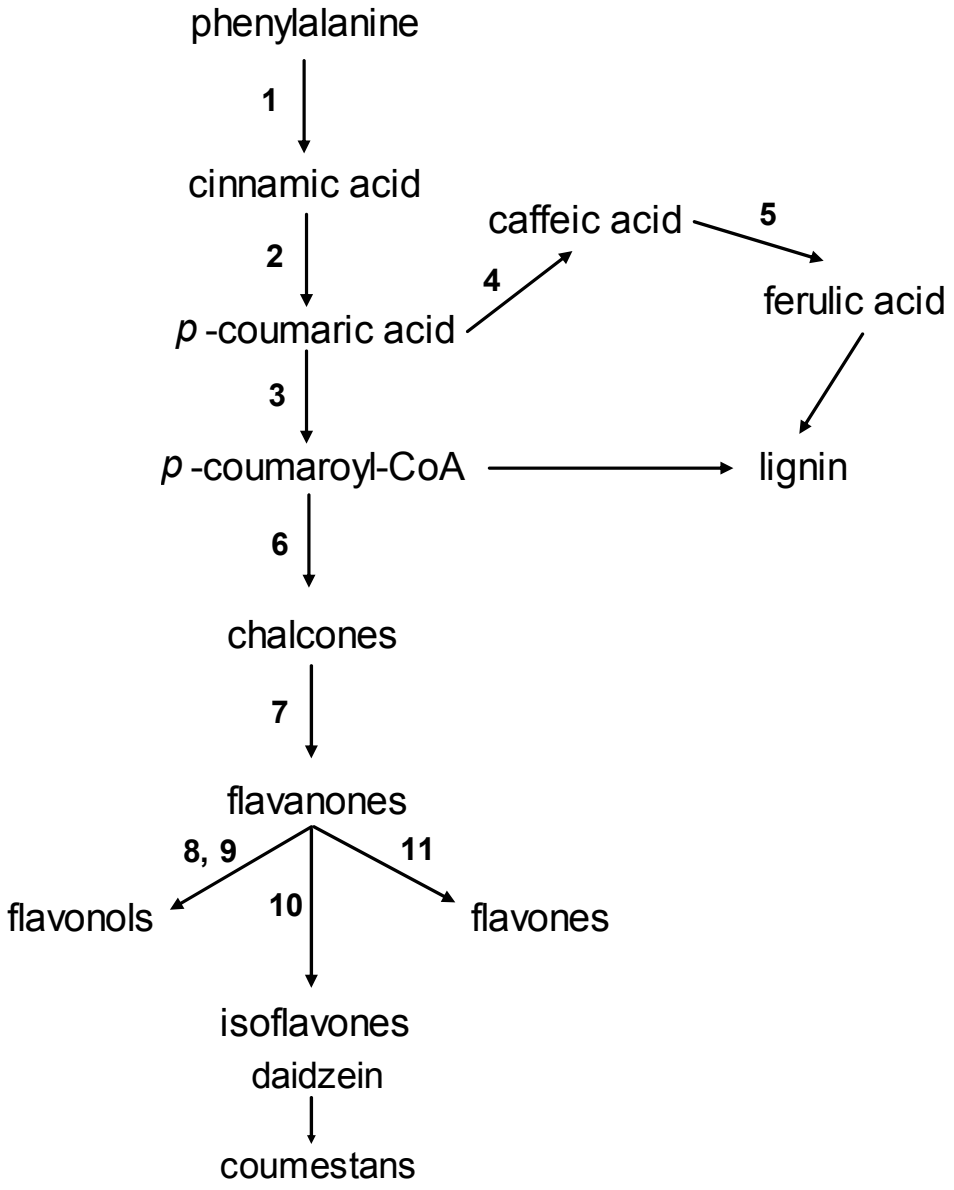

Figure 27 – Sequential diagram indicating metabolism of phenylpropanoid compounds present in mungbean seedlings. Enzymes involved include 1 = phenylalanine ammonia lyase, 2 = cinnamic acid 4-hydroxylase, 3 = 4 coumarate:CoA ligase, 4 = hydroxylase, 5 = *O*-methyltransferase, 6 = chalcone synthase, 7 = chalcone isomerase, 8 = flavanone 3-hydroxylase, 9 = flavonol synthase, 10 = isoflavone synthase, 11 = flavone synthase. Based on Heller and Forkmann (1994), Taiz and Zieger (1998a), Jung and others (2000), Croteau and others (2001), and Durango and others (2002).

During germination, decreases in the flavones could be due to reduced synthesis or inactivation of flavone synthase and activation of cinnamic acid 4-hydroxylase, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonol synthase and isoflavone synthase (Figure 27). During wounding and UV-C there could have been increases in the synthesis or activation of the enzymes involved in lignification, due to observed decreases in *p*-coumaroyl, caffeoyl and feruloyl derivatives, important lignin precursors (Table 3). Other enzymes synthesized or activated could have been chalcone isomerase and isoflavone synthase due to observed increases in flavanones and coumestans and decreases in the *p*-coumaroyl derivative (Table 3). For UV-C effect only, it is possible that flavanone 3-hydroxylase and flavonol synthase activation or synthesis increased due to observed increases in flavonols, while for wounding stress the opposite could have occurred.

In general, phenolic synthesis results during germination, wounding and UV-C treatments indicate possible lignification and phytoalexin production. Lignification has been shown to be enhanced by ROS such as hydrogen peroxide, while phytoalexin production in a similar way via ROS (Murphy and Huerta 1990, Vranova and others 2002, Ros Barcelo and others 2003, Perkovskaya and others 2004).

Even though some compounds appeared due to wounding and UV-C stresses, these compounds were already present in controls at very low amounts. After hydrolyzing (alkaline + acid hydrolysis) wound and UV-C treated samples, it was observed that the phenolic profiles showing aglycones and non-hydrolyzed glycosidic-bound phenolics were very similar, confirming absence of new phenolic synthesis and suggesting mediation by similar signal molecule(s) (Figure 28).

Conclusions

The phenolic synthesis studies indicated possible utilization of some phenolic compounds for lignification and an enhancement of phytoalexin synthesis for protection functions. In addition, the observed increases in phenolics due to exogenous hydrogen peroxide application, and the probable mediation of hydrogen peroxide on decreased

growth of UV-C treated seeds indicate this ROS to be a clear candidate for mediating phenolic synthesis responses during germination, wounding and UV-C treatments. In addition, other signaling molecules seem to be participating as well due to differential synthesis responses on flavonols and other phenolic compounds giving different phenolic profiles.

In Figure 29 we present a summary diagram of phenolic synthesis and growth of germinating mungbean seedlings in response to potential signal molecules and stresses, together with the presumed roles of the synthesized phenolic compounds. In this diagram, we propose ROS to be key signaling molecules mediating phenolic synthesis responses as well as growth regulation functions during normal germination and upon exposure to wounding or UV-C stresses.

Due to the similar effects of exogenous hydrogen peroxide on phenolic synthesis and seedling growth compared to UV-C stress, as well as similar signaling mediation during germination and UV-C treatment, the next chapter includes a detailed study on hydrogen peroxide as a potential signaling molecule mediating phenolic synthesis responses during germination and UV-C treatment.

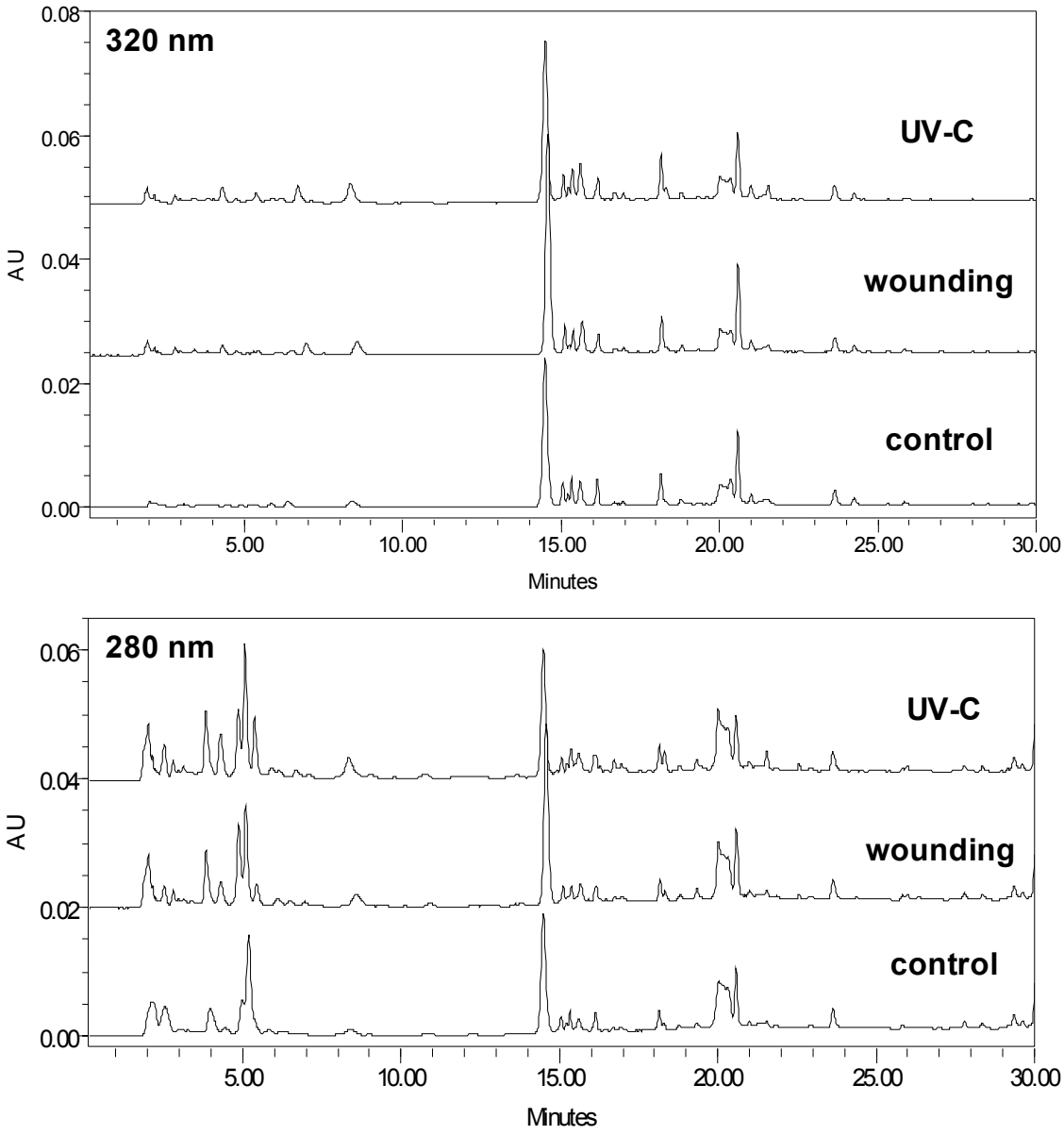

Figure 28 – HPLC profiles after alkaline + acid hydrolysis on extracts of 4 d old mungbean seeds grown at 25°C without stress or subject to wounding or UV-C stresses. AU = absorbance units. X-axis is retention time in min.

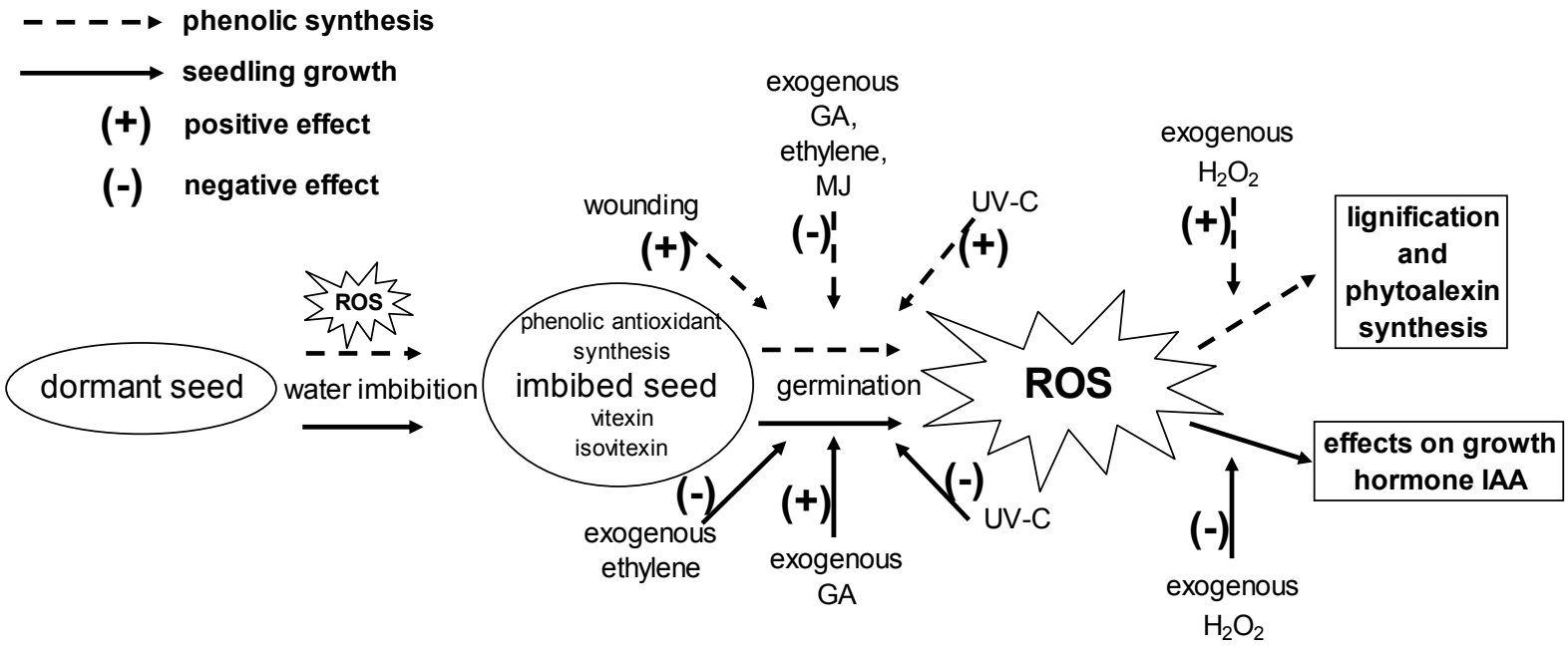

Figure 29 – The proposed central role of reactive oxygen species as mediators of phenolic synthesis and growth development of mungbean seeds during germination and upon exposure to potential signal molecules and stressors. ROS (reactive oxygen species), GA (gibberellic acid), MJ (methyl jasmonate), IAA (indole-3-acetic acid).

CHAPTER V

MECHANISM OF PHENOLIC SYNTHESIS IN MUNGBEAN SEEDS DURING GERMINATION AND UPON EXPOSURE TO UV-C STRESS

Synopsis

We determined phenolics to be important compounds during dark germination and more important for protection due to UV-C stress. Signal transduction processes for UV-C stress started with transient increases in the levels of H_2O_2 together with increases in respiration, followed by increases in phenylalanine ammonia lyase (PAL) activity, then synthesis of soluble/bound phenolics, followed by guaiacol peroxidase (POX) activity and lignin synthesis. Signal transduction for dark germination effect alone followed the same order as UV-C, but with lower increases in these parameters. The cotyledon seemed to be the main source of phenylpropanoids and/or related signals for synthesis in hypocotyl and root sections, tissues demanding precursors for lignification due to active growth. Regarding ROS, we propose that the main synthesis of soluble phenolics during dark germination and UV-C stress is mediated mainly by ROS synthesized by plasma membrane bound NADPH dependent oxidase compared to ROS from other sources such as peroxidases. Decreases in $O_2^{\cdot -}$ and increases in H_2O_2 levels due to UV-C indicate the latter species to be the potential signal mediators. Apart from mediating phenolic synthesis responses, ROS seem to be important for peroxidase activity induction and seedling growth regulation. Regarding health benefits of dark germinated and UV-C treated seeds, this study showed that germination increases the level of phenolic antioxidants, and this increase is enhanced by UV-C stress. The seed coat is a concentrated source of antioxidants only after water imbibition, while cotyledon and hypocotyl sections contribute to most of the antioxidant properties at later germination stages. Increases in lignin due to UV-C stress could contribute to the health benefit properties of germinated seeds due to its role as dietary fiber.

Introduction

From the previous chapter (Chapter IV), it was speculated that H_2O_2 could be an important mediator of phenolic synthesis during germination and UV-C stress. In this chapter we focus on the study of reactive oxygen species (ROS) as potential signal molecules of phenylpropanoid synthesis, since the mechanisms of phenylpropanoid induction due to normal seed germination and UV-C are still unclear.

During normal growth or upon exposure to an abiotic stress, plant secondary compounds including phenolics, are synthesized after a complex sequence of signal transduction processes. Responses start with a signal, either extracellular or intracellular, which is perceived by a receptor on the surface of the plasma membrane (Taiz and Zieger 1998g, Taiz and Zieger 1998h). This receptor then initiates a signal transduction network leading to activation or de novo synthesis of transcription factors responsible for regulating gene expression related to synthesis of secondary metabolites (Low and Merida 1996, Koiwa and others 1997, Morgan and Drew 1997, Taiz and Zeiger 1998g, Taiz and Zieger 1998h, Vranova and others 2002, Zhao and other 2005). In the sequence of events for phenolic synthesis important components include elicitor signals (i.e. physical nature), activated effectors (i.e. ion channels, GTP binding proteins [G-proteins], protein kinases), secondary messengers (i.e. ROS, jasmonic acid, ethylene), enzymes related to phenylpropanoid metabolism (i.e. phenylalanine ammonia lyase [PAL]) and initial (i.e. soluble phenolics) and final metabolic products (i.e. lignin) of the related phenylpropanoid enzyme-catalyzed reactions. According to Zhao and others (2005), a proposed sequence of events would be: "perception of elicitor by receptor, reversible phosphorylation and dephosphorylation of plasma membrane proteins and cytosolic proteins, cytosolic $[Ca^{2+}]_{cyt}$ spiking, plasma membrane depolarization, Cl^- and K^+ efflux/H^+ influx, extracellular alkalinization and cytoplasmic acidification, mitogen-activated protein kinase (MAPK) activation, NADPH oxidase activation and ROS production, early defense gene expression, ethylene and jasmonate production, late defense response gene expression, and secondary metabolite accumulation". In detail, the elicitor signal initiates the responses, as it is perceived by

the plasma membrane receptor. After elicitor signals are perceived by a receptor, there is activation of the effectors, which will transfer these signals to second messengers, responsible for amplifying the elicitor signal to other downstream reactions. These second messengers could call for the synthesis or activation of other messengers or could directly elicit the gene expression response. A gene activated due to a secondary messenger would code for the synthesis of enzymes related to phenolic synthesis (i.e. PAL). Upon enzymatic activation or de novo synthesis, there will be increased synthesis of phenolic compounds. These metabolites could start exerting their functions (e.g. as antioxidants, antimicrobials) or could be transformed towards the synthesis of protective cell wall barriers (i.e. lignin, suberin).

The role of ROS as secondary messengers mediating phenolic synthesis due to several abiotic stresses has been postulated by several authors as reviewed by Low and Merida (1996) and Vranova and others (2002). Gene expression related to plant defense mechanisms mediated by ROS has been shown as response to pathogen attack, chilling injury, wounding, excess light and UV-C (Murphy and Huerta 1990, Vranova and others 2002, Ros Barcelo and others 2003). Since plants need oxygen to produce energy through respiration, during the reduction of O_2 to H_2O, ROS such as $O_2^{\cdot -}$, H_2O_2, and hydroxyl radical can be formed (Vranova and others 2002). Several sources of ROS include chloroplast, cytoplasmic cytochrome P450, mitochondrial oxidases, peroxisome, endoplasmic reticulum, plasma membrane NADPH-dependent oxidase (NADPH oxidase), and apoplastic and cell wall peroxidases (Vranova and others 2002, Zhao and others 2005). Another source of ROS during UV-C stress could be the physical production of free radicals due to alterations in the atoms or molecules present in the cell, especially in water (Kovacs and Keresztes 2002). NADPH oxidase seems to be the major source of ROS during stress responses; however controversy still exists on the important contribution of ROS produced by peroxidases in the presence of NADH (Dat and others 2000, Vranova and others 2002). Regarding UV-C, it is not clear whether or not ROS mediate phenylpropanoid induction and if they do, the precise mechanism of this mediation still awaits elucidation.

Activation of NADPH oxidase and triggering of the oxidative burst seems to be mediated by Ca^{2+} influx, alkalinization of the apoplast, protein phosphorylation and translocation of the $p47^{phox}$ and $p67^{phox}$ subunits to membrane bound cytochrome b_{558} (cyt b_{558}) (Low and Merida 1996, Vranova and others 2002). NADPH oxidase is responsible for the reduction of O_2 to $O_2^{\cdot-}$, which is then transformed to H_2O_2 by superoxide dismutase (SOD) (Low and Merida 1996, Zhao and others 2005). Involvement of these radical species on local or systemic plant stress-induced defense responses has been shown. H_2O_2 could even have a direct role on the activation of NADPH-oxidase, therefore stimulating more ROS (Dat and others 2000).

By inhibiting NADPH oxidase, with compounds such as diphenyleneiodonium chloride (DPI), it is possible to determine the participation of ROS during plant defense responses. DPI binds to the flavoprotein and cyt b_{558} moieties of the reduced NADPH oxidase complex, thus inhibiting electron transfer from NADPH to O_2 (Frahry and Schopfer 1998). DPI has also been shown to inhibit the O_2-reducing activity of peroxidases producing $O_2^{\cdot-}$ and H_2O_2 in the presence of NADH (Frahry and Schopfer 1998).

Figure 30 summarizes the sequence of events of phenylpropanoid induction in response to abiotic stresses mediated by plasma membrane bound NADPH oxidase, including the inhibitor effect of DPI.

In the present chapter we elucidate some of the mechanisms by which mungbean seedlings synthesize and transform phenolics during dark germination and UV-C stresses. We evaluated the sequence of events for phenolic synthesis during dark germination and UV-C stress. Measured parameters included soluble and cell-wall bound phenolics, lignin, ROS ($O_2^{\cdot-}$ and H_2O_2), protein content, peroxidase and PAL activities, and ethylene and respiration rates (RCO_2). Measurements of antioxidant properties were also conducted as indication of the type of phenolics synthesized. Soluble and cell-wall bound phenolis, and lignin determinations were also conducted on separate seed sections (cotyledon, hypocotyl and seed coat) for determining tissue-specific roles and contributions during phenolic synthesis and transformation. NADPH-

oxidase inhibition studies with DPI were conducted for determining the role of ROS on phenolic synthesis responses during germination process and UV-C stress.

Materials and Methods

Materials

Mungbean (*Vigna radiata* L. Wilczek) seeds were purchased from Johnny's Selected Seeds (Winslow, ME, USA). Chlorogenic acid, Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). H_2O_2 was obtained from Fisher Scientific (Houston, TX, USA).

Seed germination and UV-C treatment

Mungbean seeds were allowed to imbibe water for 14 h at 25°C. Then water was removed and seeds were dark-germinated at 25°C in cheesecloth covered glass jars with 3 layers of humidified paper towels. Paper towels were kept moist by spraying with sterile water as needed. One day old (from water imbibition) mungbean seedlings were exposed to 240W UV-C for 40 min.

For most assays, 6 seeds were used per replicate, with 3 to 4 replicates per assay and 3 to 6 repetitions per replicate.

Inhibition studies with diphenyleneiodonium chloride (DPI)

To inhibit superoxide radical ($O_2^{\cdot-}$) production from NADPH oxidase and NADH-coupled peroxidase, 1 d old mungbean seedlings were dipped in 100 mL DPI solution (0, 25 or 250 uM) for 1.5 h. DPI was first dissolved in DMSO (dimethyl sulfoxide) (1:100 w/v) yielding 0.8% DMSO in final solution after dilution with nanopure water. Solutions without DPI also contained 0.8% DMSO. UV-C treatment (240W for 40 min) was applied immediately after DPI dip.

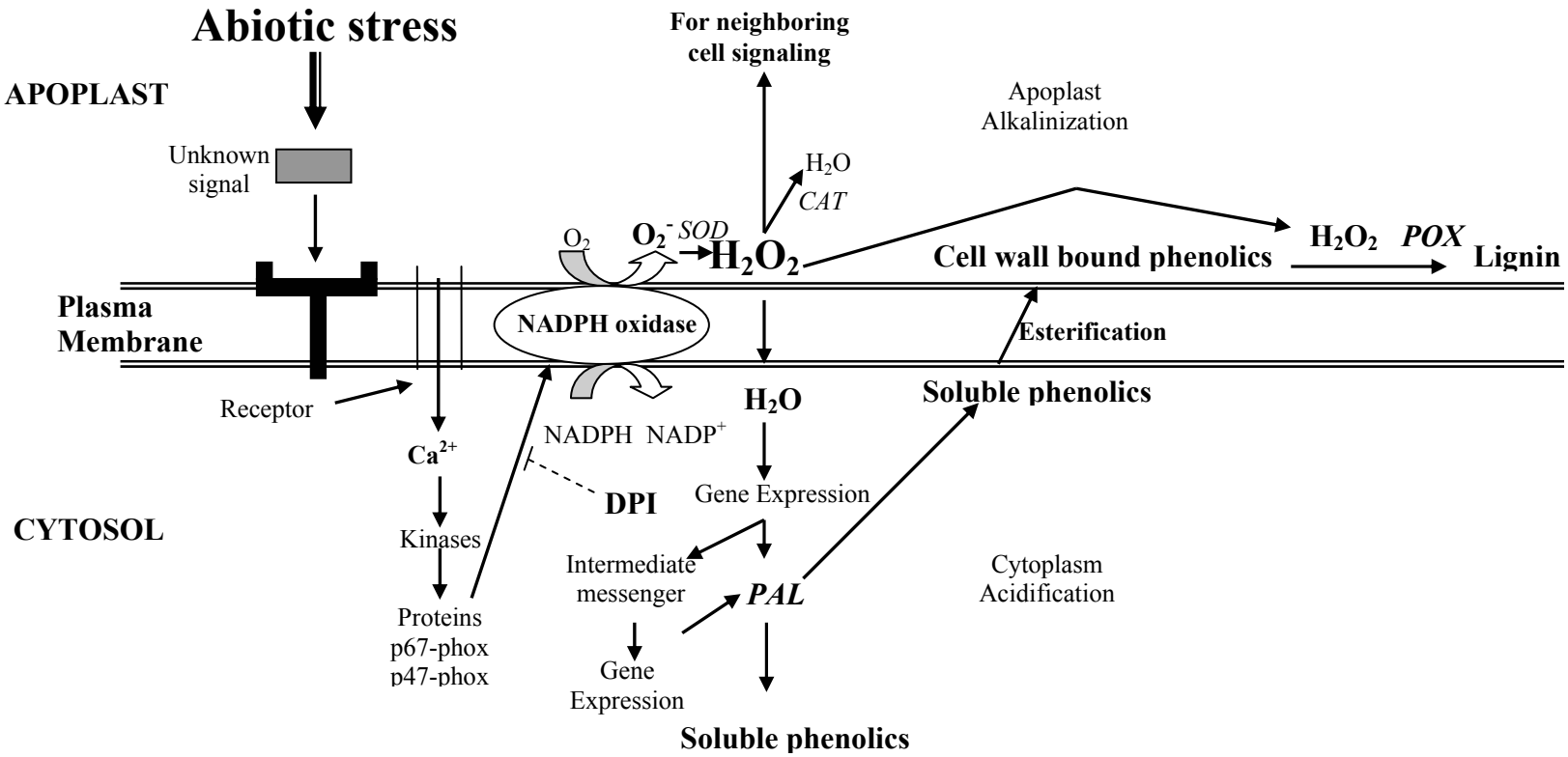

Figure 30 – Sequence of events for phenylpropanoid metabolism mediated by NADPH oxidase in response to abiotic stress. The central roles of H_2O_2, together with the target of DPI inhibition are depicted. DPI (diphenyleneiodonium chloride), SOD (superoxide dismutase), CAT (catalase), PAL (phenylalanine ammonia lyase), POX (guaiacol peroxidase).

Soluble phenolics, cell wall bound phenolics and lignin

Total soluble phenolic content of methanolic extracts was adapted from Cevallos-Casals and Cisneros-Zevallos (2003) using Folin-Ciocalteu reagent. Measurements were conducted at 725 nm in a microtiter plate reader as described in Chapter III. Total phenolics were expressed as mg chlorogenic acid equivalents (CAE) 100 g^{-1} dry basis (DB) or per seed basis (PSB), based on a standard curve.

The evaluation of cell wall bound phenolics and lignin was performed by adapting the methods of Campbell and Ellis (1992), Brinkmann and others (2002), and Ascensao and Dubery (2003). The pellet obtained after centrifuging in the assay for soluble phenolics was dried at 20°C for 12 h and used for bound phenolics and lignin content. This pellet was subjected to alkaline hydrolysis under N_2 (15 mL of 2 M NaOH for 1 h at 20°C). After 1 h, samples were centrifuged for 15 min at 29,000g. Then, the supernatant was neutralized with 6N HCl and bound phenolics were measured using the same procedure as for soluble phenolics.

The pellet obtained after centrifugation for bound phenolics was used for lignin determination. First, the pellet was washed with 20 mL nanopure water and centrifuged for 15 min at 29,000g. Supernatant was discarded and this procedure was repeated with methanol. After methanol was removed, the pellet was placed in an aluminum dish and dried inside a vacuum oven for 20 h at 65°C. Then, 10 mg of the dry pellet was incubated at 70°C for 30 min in 1 mL of 25% acetyl bromide in acetic acid. Then the solution was cooled down with ice water and 0.9 mL of 2 M NaOH, 0.1 mL of 7.5 M hydroxylamine hydrochloride and 8 mL acetic acid were added. Subsequently, samples were centrifuge at 12,000g for 5 min. Finally, the absorbance of the supernatant was read at 280 nm. A reagent blank was ran with every set of estimations. The lignin content was calculated using the specific absorption coefficient 20 g^{-1} L cm^{-1} for lignin and expressed as % lignin in dry matter.

Total antiradical capacity (TAC)

TAC of phenolic compounds was adapted from Brand-Williams, Cuvelier, and Berset (1995) to be used in a microtiter plate reader as described in Chapter III. Thirteen μL of methanolic sample extract (equivalent volume of methanol for the blank) were mixed with 247 μL of DPPH solution (98.9 uM in methanol) inside each well of a 96-well flat bottom microtiter plate (Costar #3595, Corning, Inc., Corning, NY). Plates were tightly sealed with several layers of parafilm to prevent evaporation, placed in the dark at 20 °C and read at 515 nm after 20 h. The change in absorbance was used and results were expressed as ug Trolox equivalents g^{-1} DB, from a standard curve.

Measurements of soluble phenolics, cell wall bound phenolics, lignin and TAC were conducted on whole seedlings as well as in 3 separate seed sections labeled as "cotyledon", "hypocotyl" and "seed coat". At imbibition stage, "cotyledon" corresponded to whole seed without seed coat. At stage A (1 to 3 days after imbibition) of Figure 31, "cotyledon" corresponded to section 2 without seed coat. At stage B (4 to 6 days after imbibition), "cotyledon" corresponded to sections 6 + 7 (Figure 31). At stage A (1 to 3 days after imbibition), "hypocotyl" corresponded to section 1. At stage B (4 to 6 days after imbibition), "hypocotyl" corresponded to sections 3 + 4 + 5 (Figure 31). Seed coat at all stages corresponded to section 8 of Figure 31.

Hydrogen peroxide (H_2O_2) content

H_2O_2 was assayed using a modification to the method of Beckett and others (2004). This assay measures H_2O_2 levels based on the oxidation of ferrous iron in acid solution and the formation of a colored complex between the ferric iron generated and xylenol orange (Gay and Gebicki 2000). Two reagent mixtures were prepared, reagents A and B. Reagent A contained 25 mM $FeSO_4$, 25 mM $(NH_4)SO_4$ and 2.5 M of H_2SO_4, while Reagent B 125 mM xylenol orange and 100 mM sorbitol. A working reagent was prepared by combining reagents A and B (1:100 v/v). For each replicate, 6 mungbean seeds were homogenized with 10 mL reagent mixture (Blanks consisted of equivalent water volume to that of seeds). Samples were centrifuged at 19,800g for 15 min at 22°C

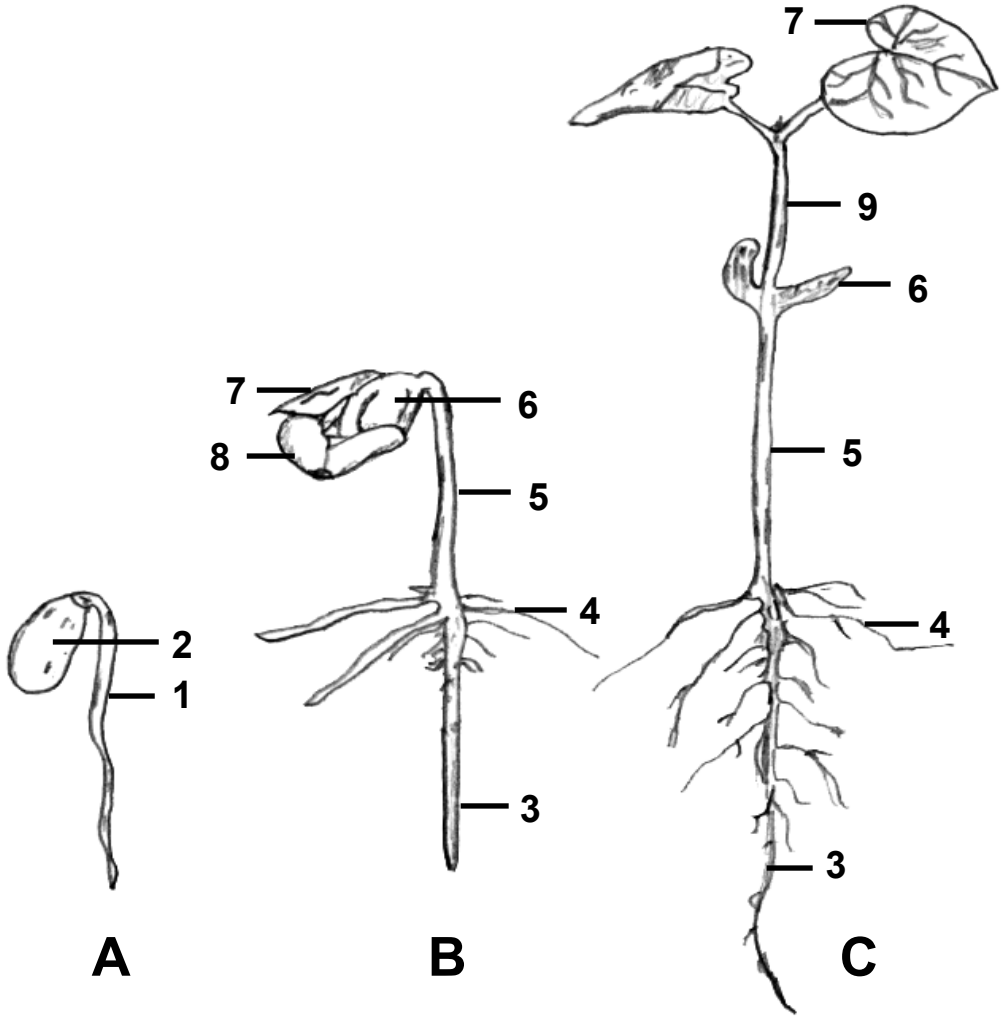

Figure 31 – Diagram of growth development of mungbean seeds at 25°C and description of its sections. Growth stages after water imbibition; A: day 1 to 3, B: day 4 to 6, C: day 6 to 10. Sections; 1: hypocotyl + primary root, 2: cotyledon + seed coat, 3: primary root, 4: secondary (branch) root, 5: hypocotyl, 6: cotyledon, 7: leaf, 8: seed coat, 9: epicotyl.

and the absorbance of the supernatant was read at 560 nm. Total reaction time from homogenization to reading was 30 min. Results were expressed as nmol H_2O_2 g^{-1} DB, based on a standard curve.

Superoxide ($O_2^{\cdot-}$) radical production

Extracellular production of $O_2^{\cdot-}$ radicals was estimated based on Beckett and others (2004) with slight modifications. $O_2^{\cdot-}$ causes the oxidation of epinephrine to adrenochrome, which exhibits an absorption maximum at 480 nm with an extinction coefficient of 4020 M^{-1} cm^{-1} (Misra and Fridovich 1972). 2.5 g of mungbean seedlings were combined with 40 mL of 1 mM epinephrine (pH 7.0) in a flask and shaken at 120 RPM for 15 min in the dark at 20°C. The absorbance of the incubation solution was measured at 480 nm after removing turbid components with 0.2 um syringe filters. Production of $O_2^{\cdot-}$ radicals was expressed as umol $O_2^{\cdot-}$ g^{-1} DB h^{-1}.

Ethylene production and respiration rate ($R$$CO_2$) measurements

Mungbean seeds (20 g) grown inside humidified 940 mL glass jars were ventilated, jars sealed and allowed to accumulate ethylene and CO_2 (<1% to avoid toxic effects) for 1 h. Then, 1 mL sample volume was withdrawn with a syringe. Ethylene content was measured by injecting the gas sample into a gas chromatograph (Photovac 10S Plus, Waltham, MA, USA) equipped with a Total VOC (Volatile Organic Compound) detector and an encapsulated capillary column together with ultra zero air as the carrier gas. Ethylene production was reported as pmoles ethylene g^{-1} DB h^{-1} from a standard curve. For $R$$CO_2$, 1 mL samples were injected into a Horiba CO_2 infrared gas analyzer (model PIR-2000, Horiba Instruments Inc., Irvine, CA, USA). $R$$CO_2$ was expressed as mL CO_2 kg^{-1} DB h^{-1}. Measurements were done in triplicate with 3 repetitions per replicate.

Phenylalanine ammonia lyase (PAL) activity

PAL activity was measured by adapting the methods described by Ke and Saltveit (1986) and Vidhyasekaran and others (2002), with some modifications. Six seeds were homogenized with 10 mL cold 0.1 M borate buffer (pH 8.8, containing 5 mM 2-mercaptoethanol). Samples were filtered with 4 layers of cheesecloth and centrifuged for 10 min at 5,000g (2 °C). The supernatant (0.25 mL) was combined with 3.75 mL 0.01 M borate buffer (pH 8.8, containing 5 mM 2-mercaptoethanol) and 1.0 mL of 0.05 M L-phenylalanine (1.0 mL water as blank) and incubated at 40 °C. Absorbances at 290 nm of samples minus blanks were recorded at 5 and 65 min. PAL activity was expressed in terms of umol *t*-cinnamic acid formed g^{-1} DB h^{-1}, based on a standard curve.

Guaiacol peroxidase (POX) activity

POX activity was adapted from Ramanathan and Vidhyasekaran (1997). Six seeds were homogenized with 20 mL of cold 0.1 M sodium phosphate buffer (pH 6.5). Samples were filtered with 4 layers of cheesecloth and centrifuged for 10 min at 5,000g (2°C). The supernatant (0.05 mL) was diluted with 2.6 mL buffer and reacted with 0.25 mL 0.1 M guaiacol and 0.1 mL 0.25% H_2O_2 inside a quartz cuvette. Absorbance readings at 420 nm were taken every 20 s for 1 min at 20 °C and reaction rate calculated from the linear regression fit of the curve. One unit (U) of POX activity was defined as the increase in absorbance at 420 nm min^{-1}. Results were expressed in U g^{-1} DB.

Total soluble protein

Soluble protein content was measured according to the method of Bradford (1976). Eight uL of the enzyme extract used for POX activity (8 uL buffer as blank) were mixed with 240 ul Bradford Reagent in a 96-well microtiter plate. Samples were incubated at 20°C for 10 min and the absorbance read at 595 nm in a microtiter plate reader. Protein concentration was determined by comparing the net absorbance values against a standard curve prepared with bovine serum albumin (BSA).

Analysis of variance

One-way analysis of variance (ANOVA) was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Means were compared with Duncan's Multiple Range Test at $\alpha = 0.01$ or 0.05.

Results and Discussion

Figures 32 through 49 and Tables 4 and 5 show changes in the different parameters tested (fresh and dry seed weights, soluble and cell wall-bound phenolics, lignin, $O_2^{\cdot-}$, H_2O_2, POX and PAL activities, protein content, ethylene production and respiration rate) during dark germination and in response to UV-C stress. These changes would indicate a physiological response of the seed to dark germination and/or UV-C signals towards activation of phenylpropanoid metabolism. Figure 32 shows pictures of control and UV-C treated mungbean seeds 2 and 4 days after imbibition. At day 2 the hypocotyl was well developed and at day 4 there was development of secondary roots from the primary root (Figures 31, 32). Control seeds tended to grow whiter and more robust, while UV-C treated cells developed some reddish brown pigmentation on hypocotyl and cotyledon surfaces, especially on UV-C exposed cotyledon surfaces lacking protection from the seed coat (Figure 32). This browning intensified at day 4 and could be related to higher phenolic synthesis and higher oxidation of phenolic compounds by polyphenol oxidases and peroxidases due to stress-induced phenylpropanoid synthesis (Cantos and others 2001b). Appearance of reddish brown lesions has been shown to appear on the surface of UV-C irradiated *Cucurbita pepo* fruit tissues (Erkan and others 2001).

Changes in seed weight

Figure 33, shows that fresh seed weight increased 381% throughout germination for control and 319% for UV-C treated mungbean seeds. Three days after treatment, fresh seed weight of UV-C treated seeds was 18% lower ($p\text{-value}<0.05$) than that of control seeds (Figure 33). Lower increases in UV-C treated seeds could be due to

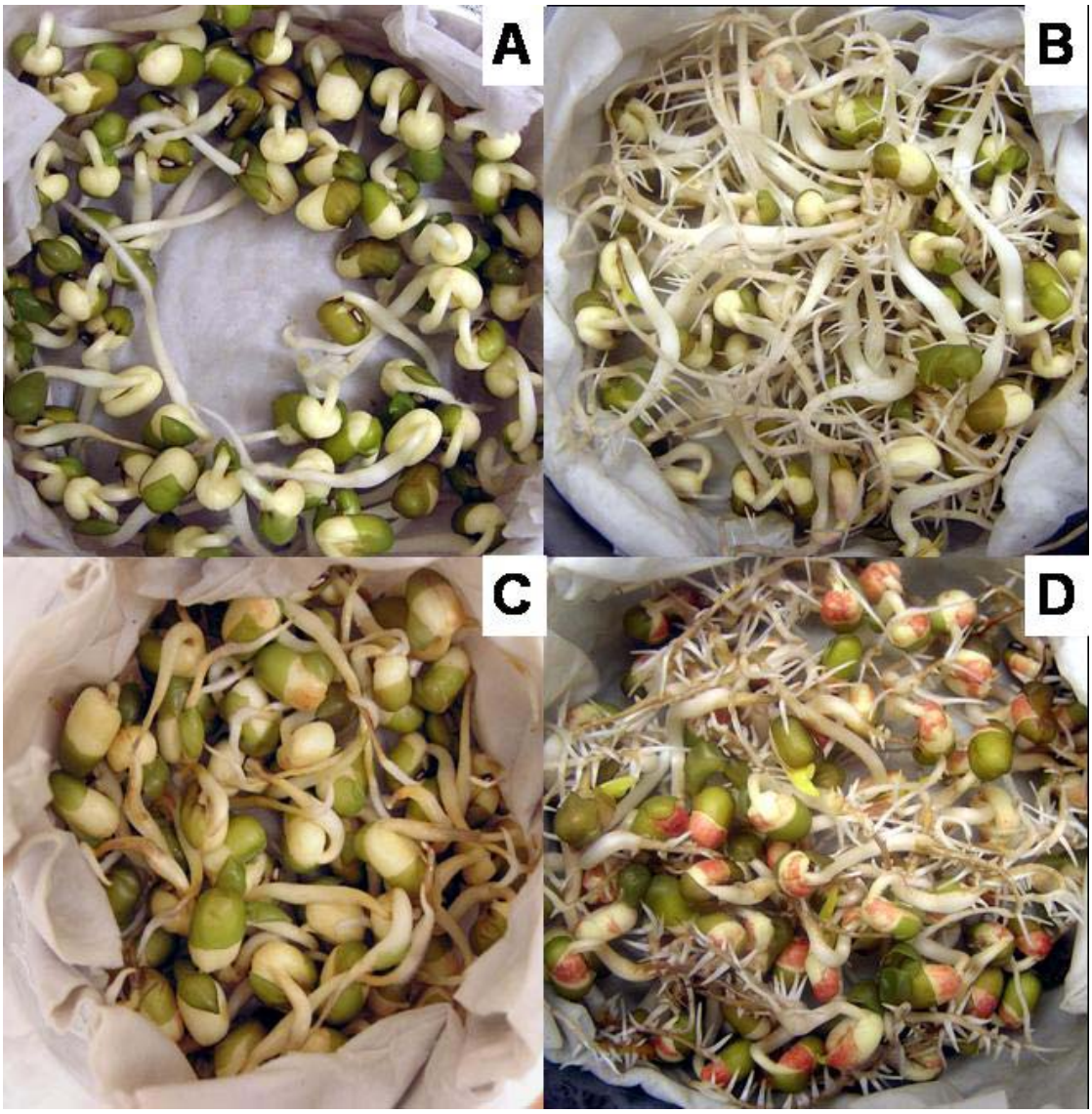

Figure 32 – Pictures of control and UV-C irradiated mungbean seeds grown at 25°C. A and B: control; C and D: UV-C; A and C: day 2 after water imbibition; B and D: day 4 after water imbibition.

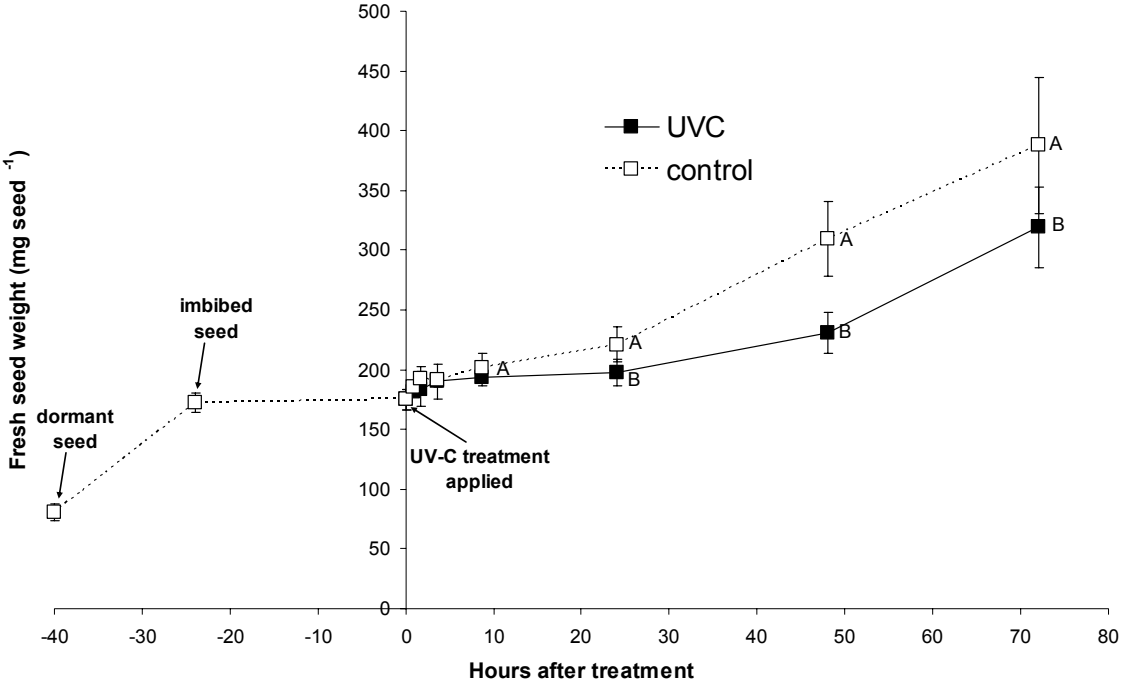

Figure 33 – Changes in fresh seed weight of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 6.

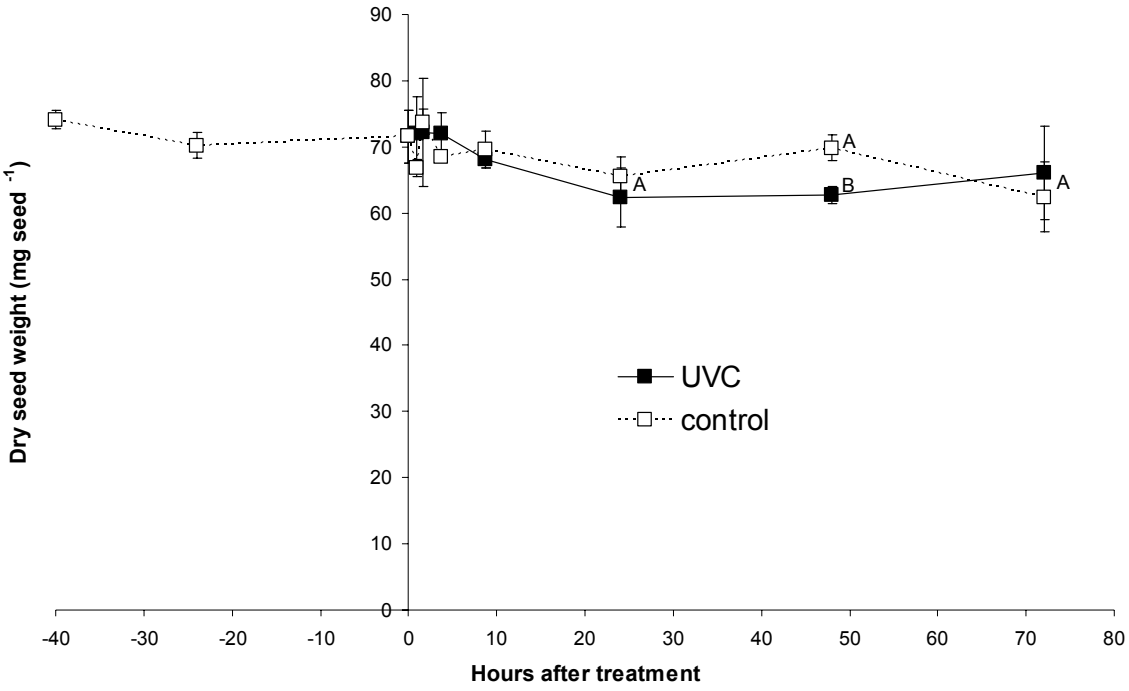

Figure 34 – Changes in dry seed weight of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

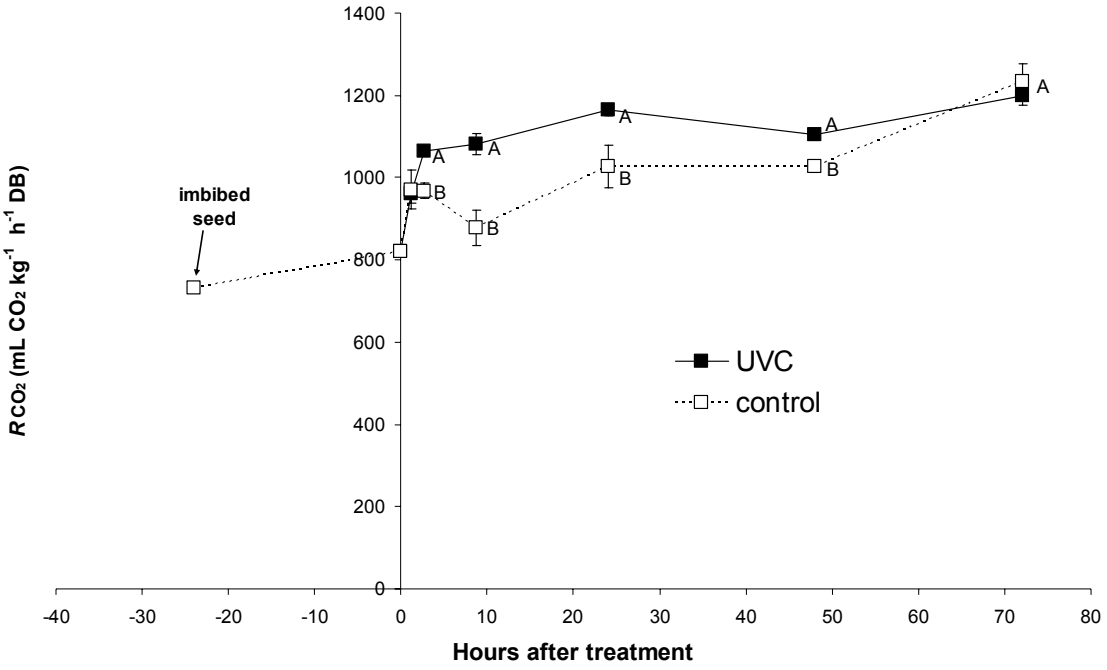

Figure 35 – Changes in respiration rate of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

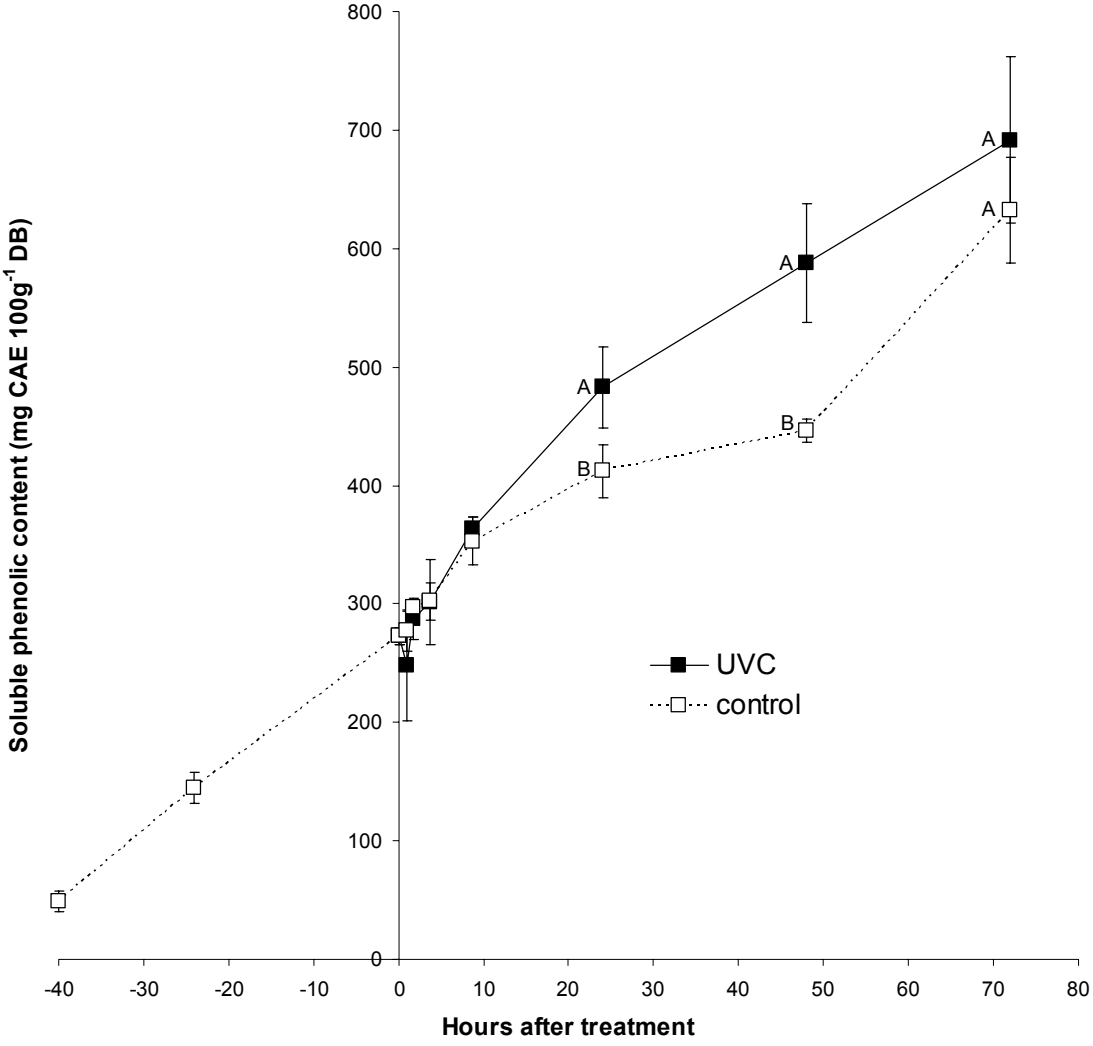

Figure 36 – Changes in soluble phenolic content of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

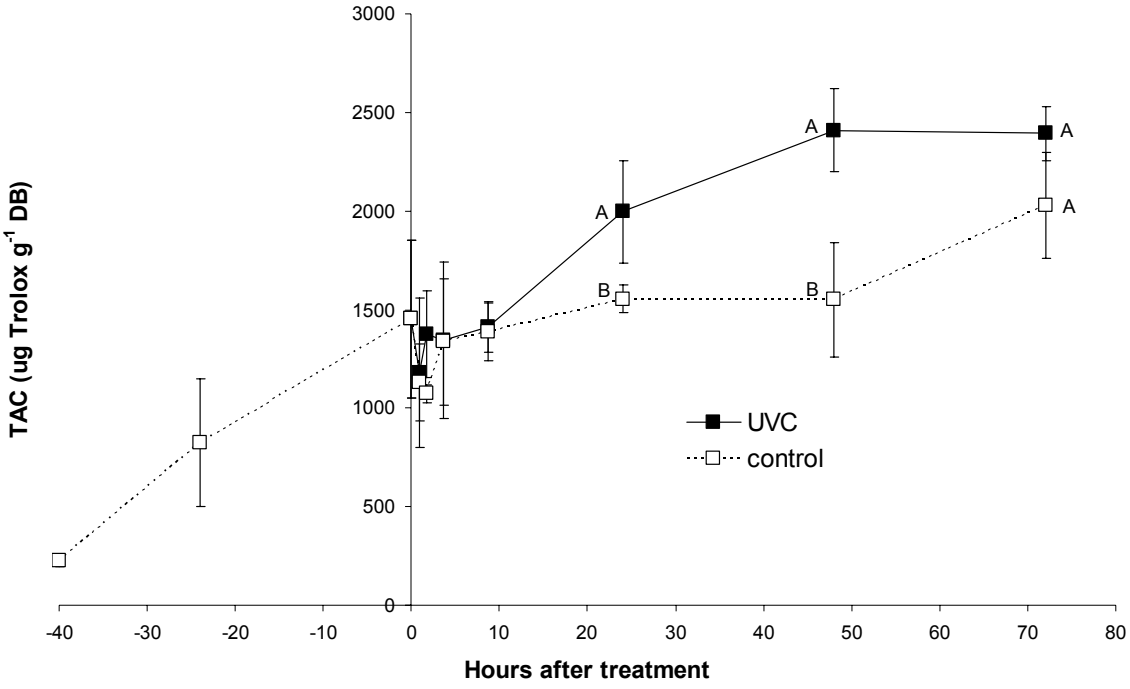

Figure 37 – Changes in antioxidant activity of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

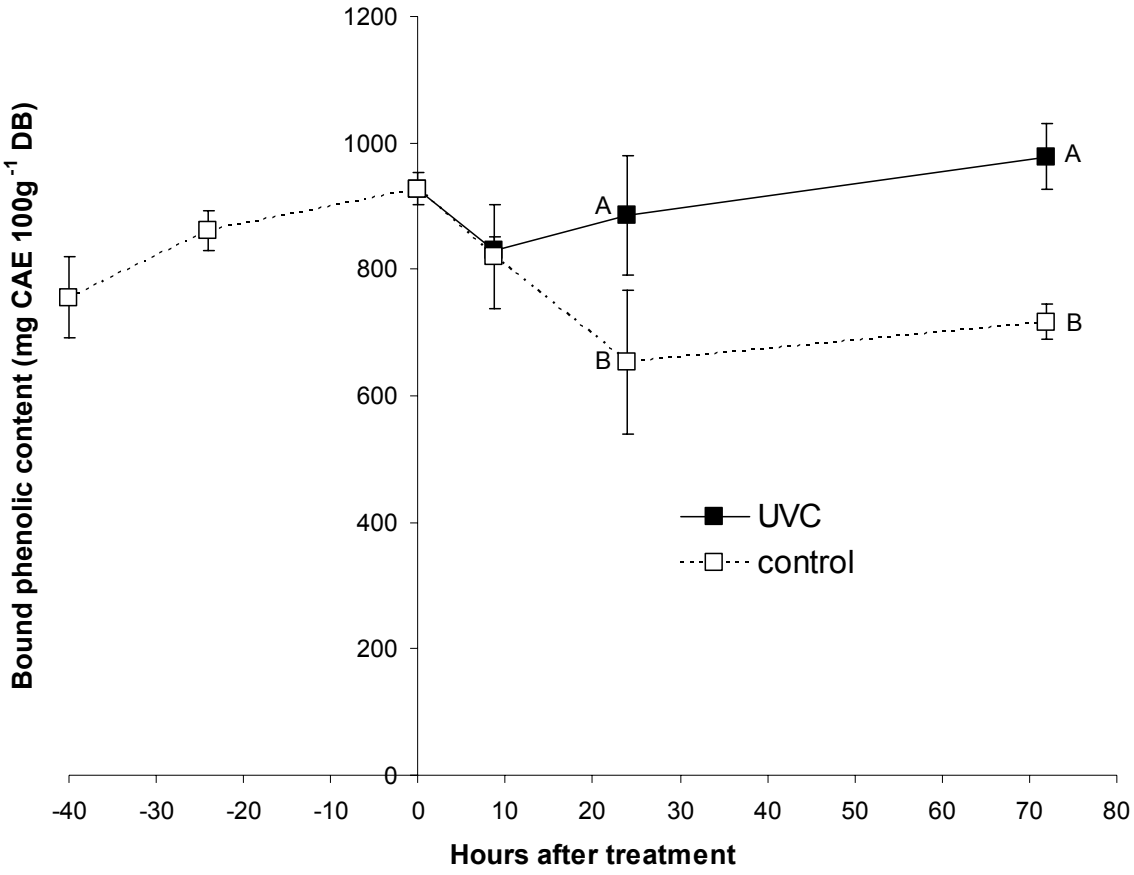

Figure 38 – Changes in cell wall bound phenolic content of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within and across the different germination times evaluated are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

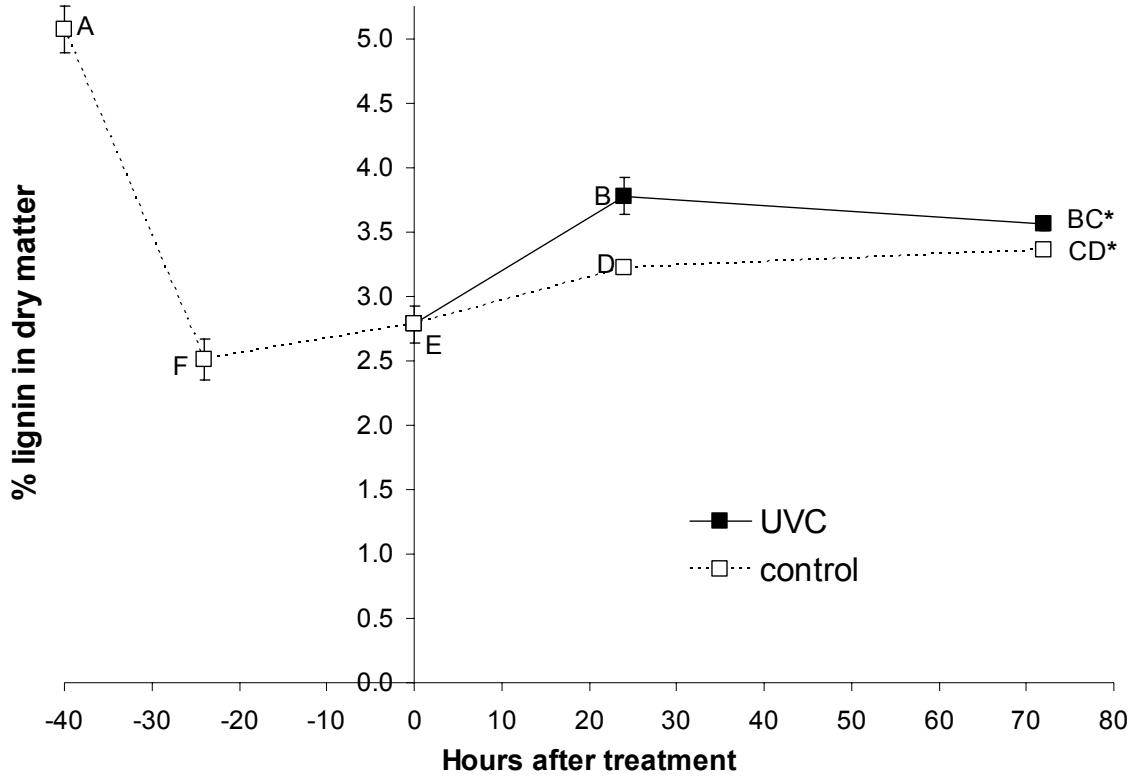

Figure 39 – Changes in lignin content of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within and across the different germination times evaluated are not significantly different (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 4. Samples with an asterisk (*) are significantly different (α = 0.05 with Duncan test) from each other when ran independent from the rest.

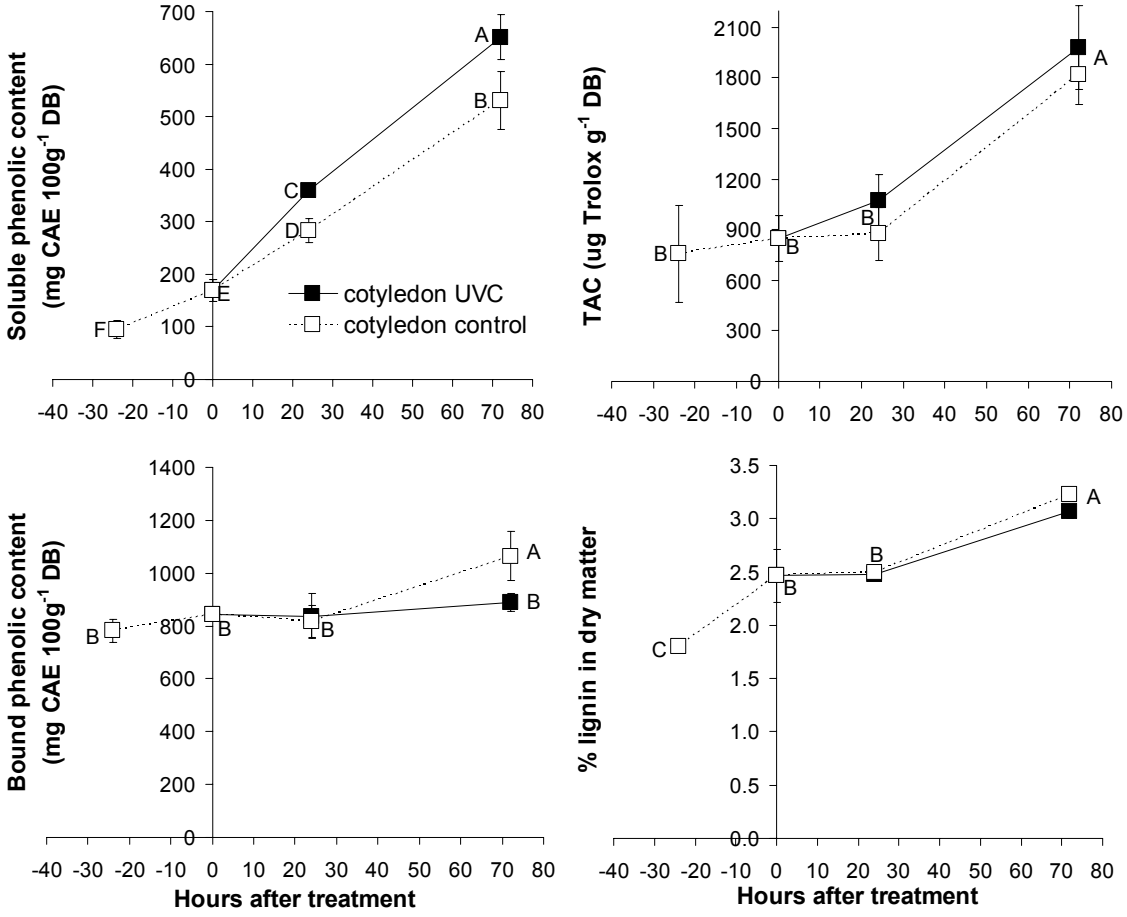

Figure 40 – Changes in soluble and bound phenolics, antioxidant activity and lignin in control and UV-C irradiated mungbean cotyledon sections during the first 112 h of dark germination at 25°C. Similar letters within and across the different germination times evaluated are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

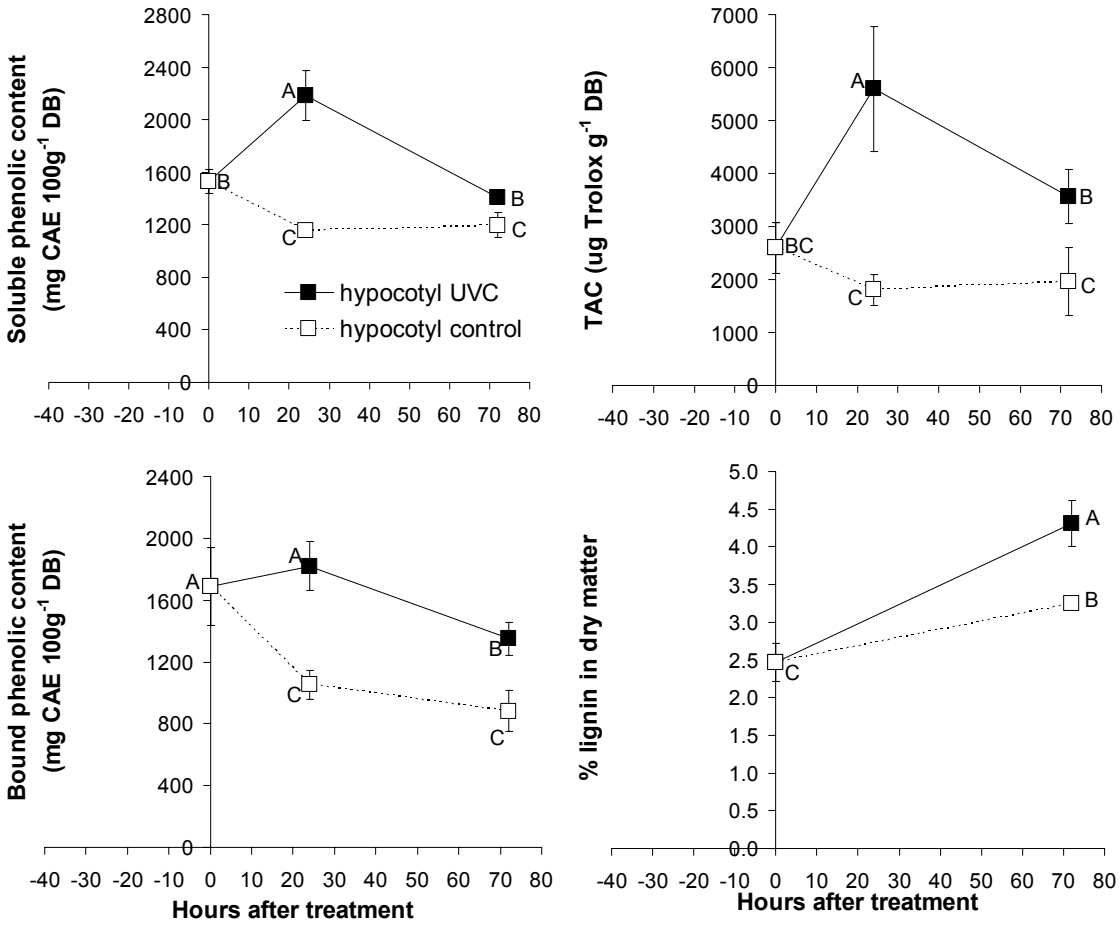

Figure 41 – Changes in soluble and bound phenolics, antioxidant activity and lignin in control and UV-C irradiated mungbean hypocotyl sections during the first 112 h of dark germination at 25°C. Similar letters within and across the different germination times evaluated are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

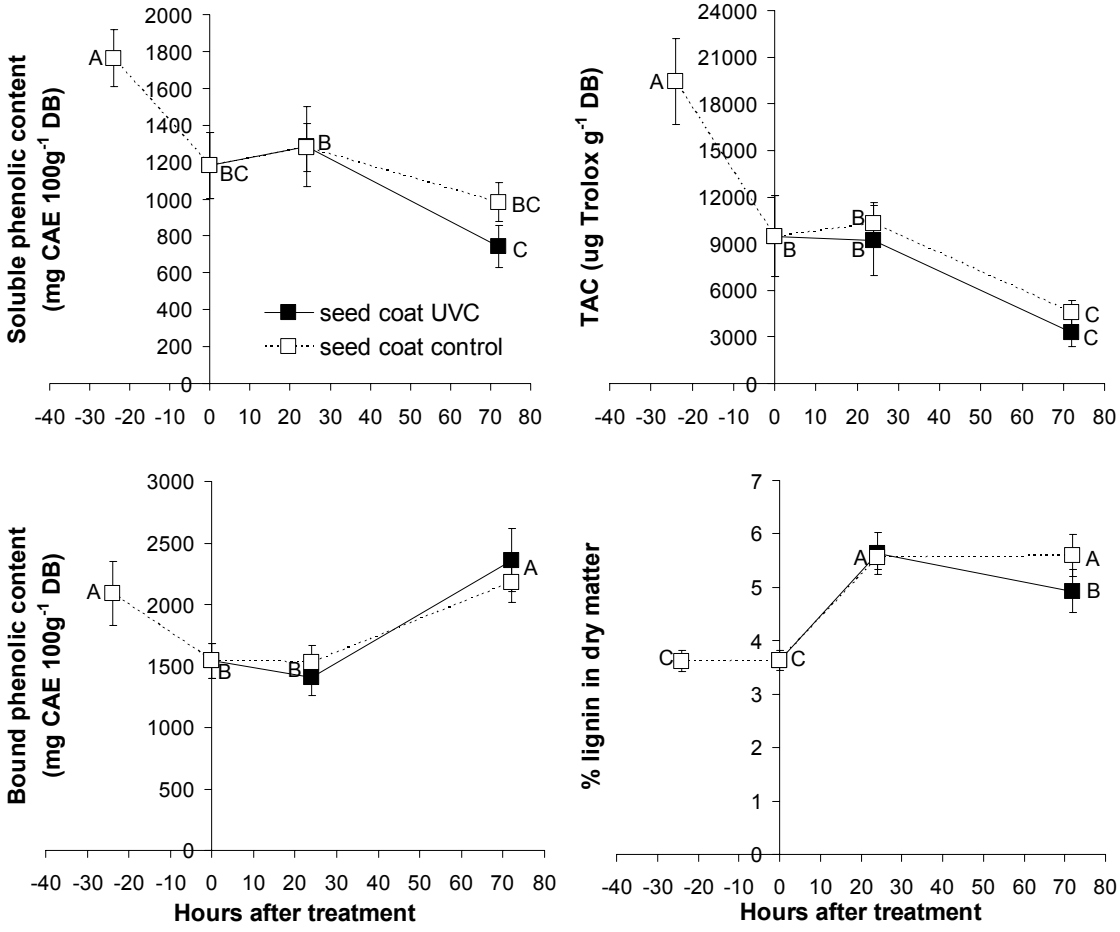

Figure 42 – Changes in soluble and bound phenolics, antioxidant activity and lignin in control and UV-C irradiated mungbean seed coat sections during the first 112 h of dark germination at 25°C. Similar letters within and across the different germination times evaluated are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

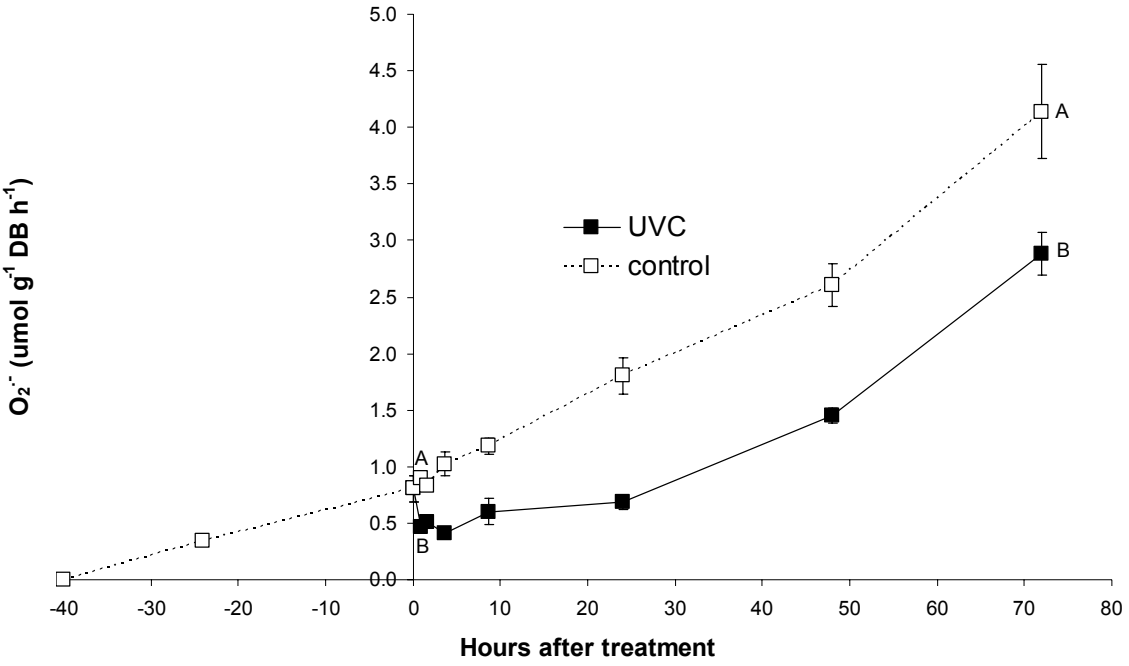

Figure 43 – Changes in superoxide radical production of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

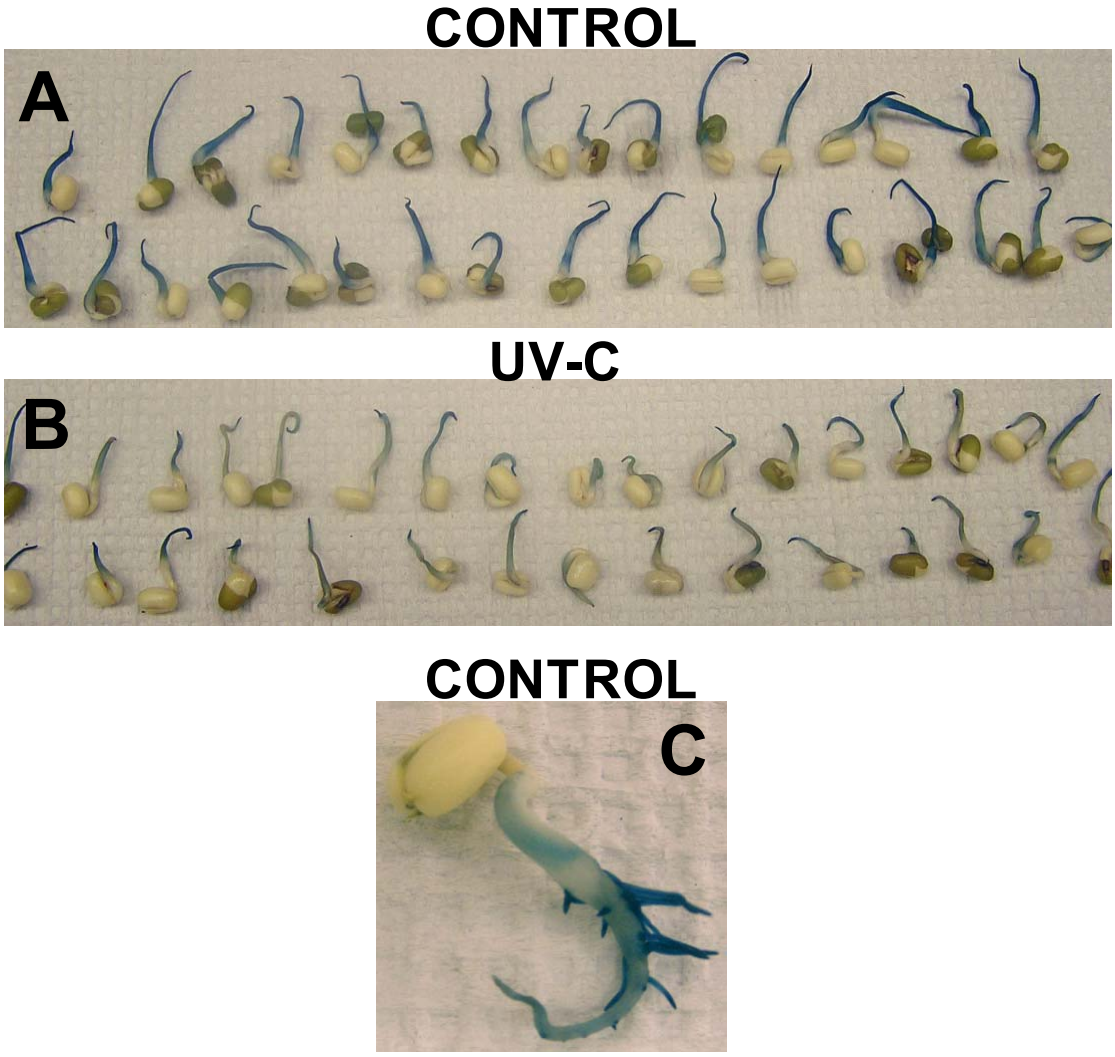

Figure 44 – Pictures showing ferric iron-xylenol orange staining for the in situ detection of H_2O_2 in control and UV-C irradiated mungbean seeds. A: control mungbean seeds 3 days after imbibition; B: UV-C irradiated mungbean seeds 3 days after imbibition; C: control mungbean seeds 4 days after imbibition.

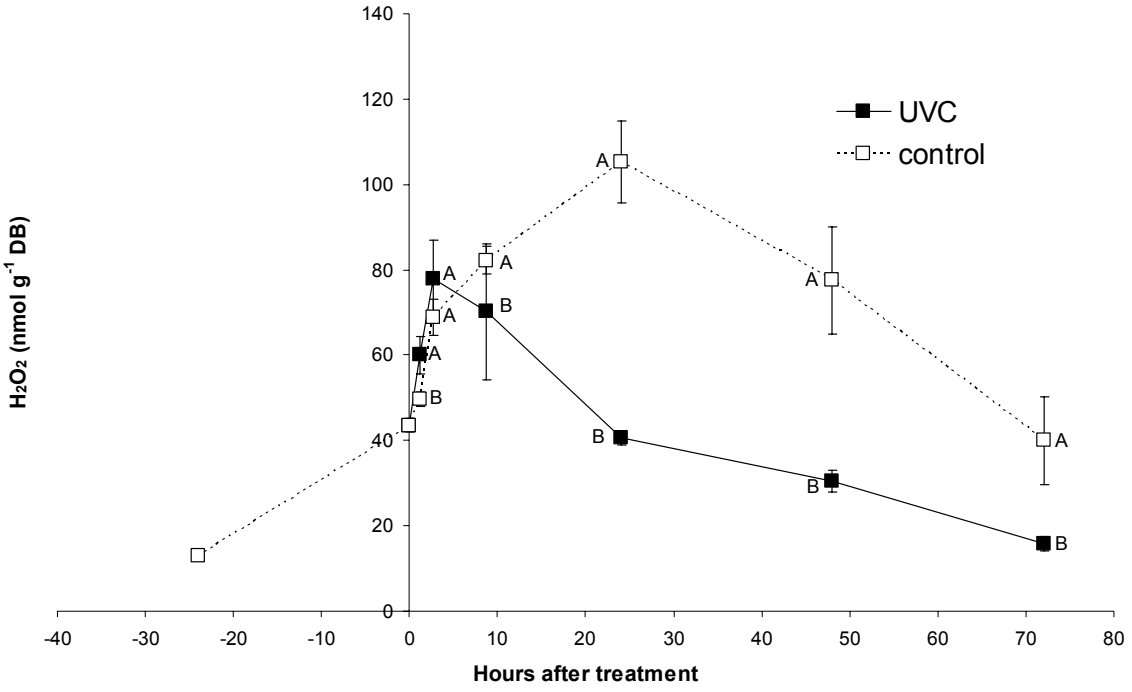

Figure 45 – Changes in hydrogen peroxide content of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

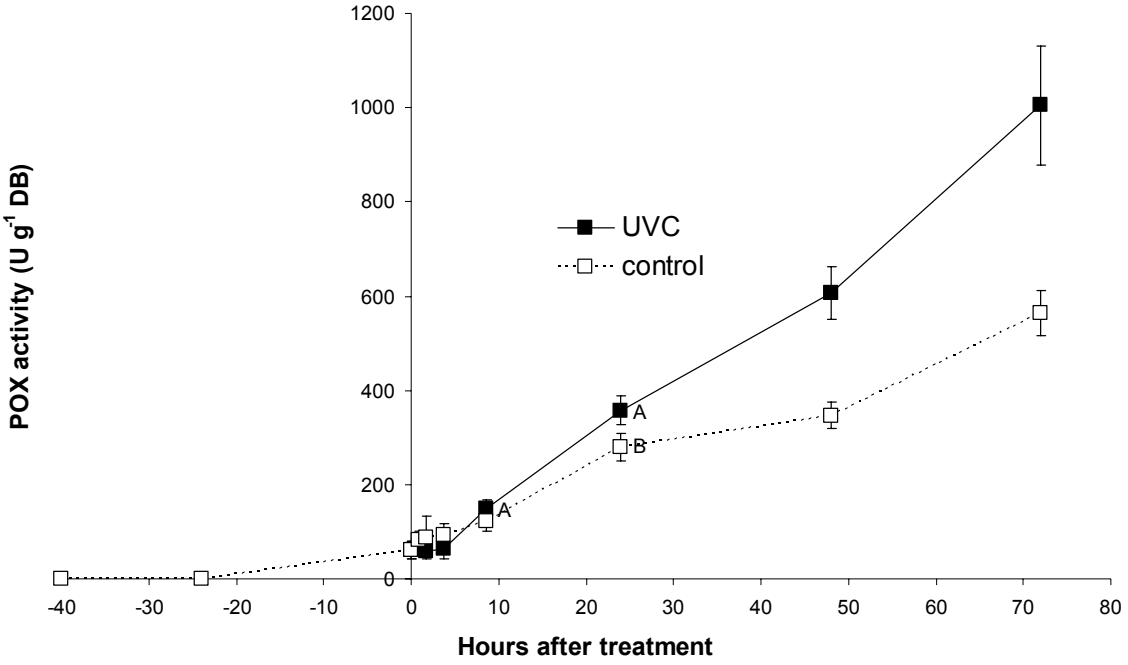

Figure 46 – Changes in guaiacol peroxidase activity of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

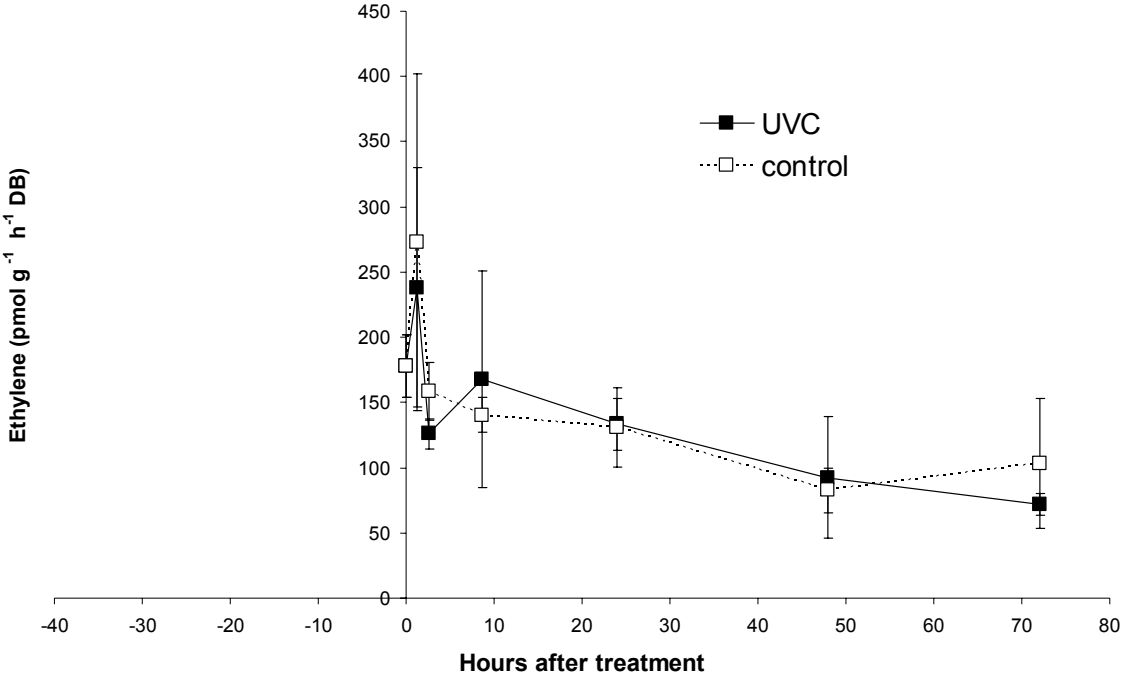

Figure 47 – Changes in ethylene production of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. No significant differences ($\alpha = 0.05$ with Duncan test) were found between controls and UV-C treatments within the same germination time. Data shows the average ± standard deviation, n = 3.

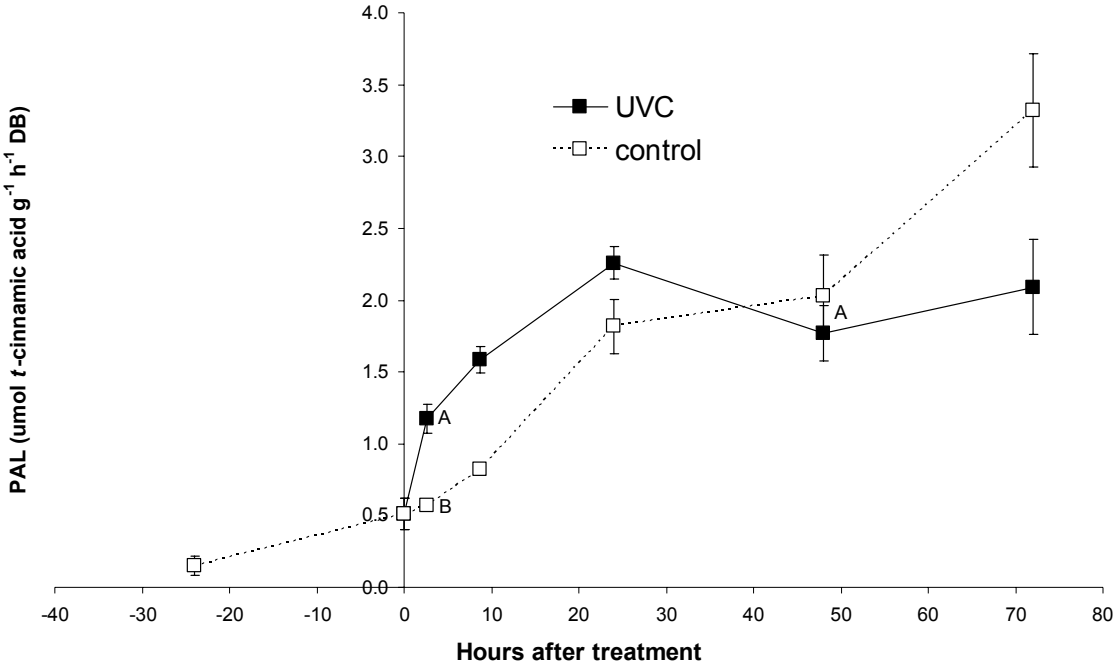

Figure 48 – Changes in phenylalanine ammonia lyase activity of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

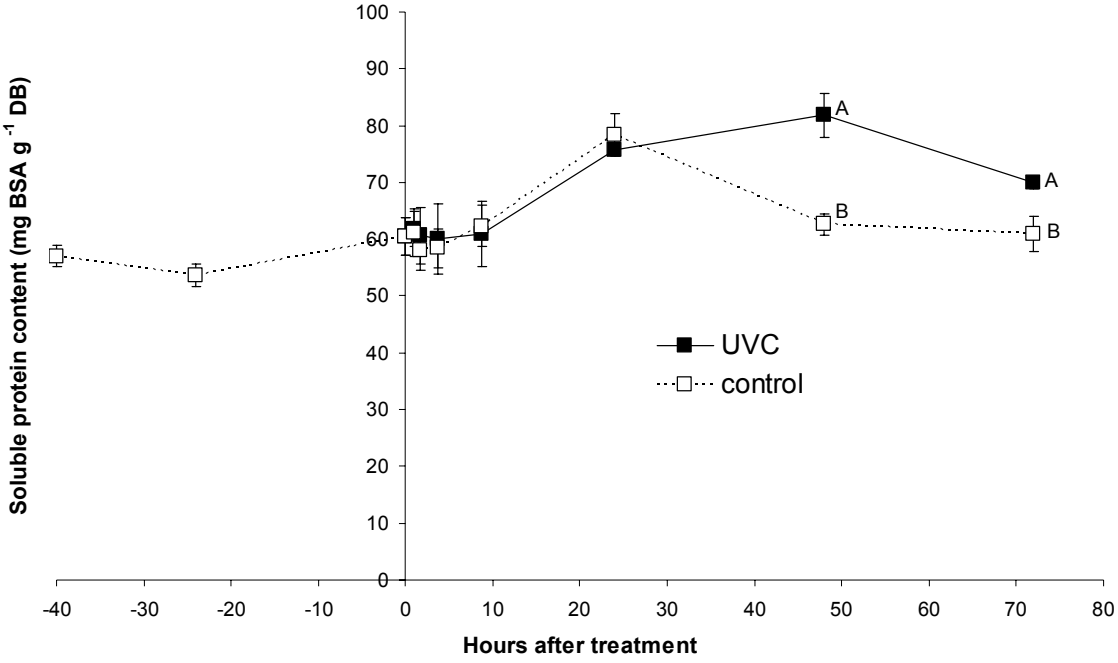

Figure 49 – Changes in soluble protein content of control and UV-C irradiated mungbean seeds during the first 112 h of dark germination at 25°C. Similar letters within the same germination time are not significantly different ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 3.

Table 4 – Changes in dry seed weight in control and UV-C irradiated mungbean cotyledon, hypocotyl and seed coat sections at different germination stages and their contributions to total dry seed weight of whole seed.

	Growth stage	Dry weight (mg)				% dry weight of whole seed		
		whole seed	cotyledon	hypocotyl	seed coat	cotyledon	hypocotyl	seed coat
Control	dormant seed	74 ± 1.4						
	imbibed seed	70 ± 2.0	73 ± 4.1		5.7 ± 0.8	92.8		7.2
	1 d after imbibition	72 ± 4.0	60 ± 4.7	4 ± 0.4	8.2 ± 2.4	82.6	6.1	11.3
	2 d after imbibition	66 ± 3.0	55 ± 2.1	10 ± 0.6	5.4 ± 0.9	78.2	14.0	7.8
	4 d after imbibition	62 ± 5.2	33 ± 5.4	23 ± 3.9	4.7 ± 0.7	54.5	37.8	7.7
UV-C	2 d after imbibition	62 ± 4.4	58 ± 2.3	5 ± 0.7	5.2 ± 0.5	85.2	7.2	7.6
	4 d after imbibition	66 ± 7.0	34 ± 4.4	17 ± 2.4	5.0 ± 0.8	61.0	30.2	8.8

Values for weight indicate average ± standard deviation, n = 4.

Table 5 – Contributions of soluble and bound phenolics, antioxidant activity and lignin for control and UV-C irradiated cotyledon, hypocotyl and seed coat sections to whole seed at different germination stages.

Growth stage	% contribution of soluble phenolics in whole seed			% contribution of TAC in whole seed			% contribution of bound phenolics in whole seed			% contribution of lignin in whole seed		
	COT	HYP	SC	COT	HYP	SC	COT	HYP	SC	COT	HYP	SC
imbibed seed	46.6		53.4	33.3		66.7	82.8		17.2	86.5		13.5
1 d after imbibe	45.8	22.3	31.9	35.6	8.1	56.3	71.5	10.6	17.9	78.4	5.8	15.8
2 d after imbibe	54.9	28.0	17.2	39.4	14.5	46.1	70.6	16.2	13.2			
4 d after imbibe	47.4	45.0	7.6	47.6	35.5	16.8	53.6	30.8	15.6	51.5	35.9	12.7
2 d after imbibe	63.7	22.4	13.9	45.4	20.0	34.6	75.0	13.7	11.2			
4 d after imbibe	58.9	35.6	5.5	46.9	41.8	11.2	46.9	35.2	17.9	51.9	36.1	12.0

COT = cotyledon, HYP = hypocotyl, SC = seed coat

possible light and UV effects on growth and development by affecting the plant growth hormone regulator indole-3-acetic acid (IAA) (Lumsden 1997, Chen and others 2002, Jayakumar and others 2003), or to probable changes in water absorption/desorption rates. It has been shown that light reduces mungbean hypocotyl growth due to IAA inactivation by light activated peroxidases (Chen and others 2002). In another work, Jayakumar and others (2003) observed that UV-B radiation inhibited development and dry matter yields. Other yield studies have determined that seed yields in linseed and pea may be reduced by moderate increases in UV-B (Corlett and others 1997). In addition, since UV-C treated seeds synthesized more phenolics than non-treated seeds, it is possible that these compounds acted as germination inhibitors (Ogawa and Iwabuchi 2001). As was mentioned before (Chapter II), the precise mechanism of action of UV-induced reduction of growth and dry matter in plants needs further elucidation.

Seed weights on a dry weight basis for UV-C treatment and control, showed a tendency to decrease throughout the tested germination period (16% decrease for control from dry seed stage) (Figure 34). These decreases in seed weight (DB) could be related to increases in seed respiration (Figure 35), therefore transforming seed carbohydrates into released carbon dioxide (Siedow and Day 2001). Differences in seed weight between UV-C treated and non-treated seeds were inconclusive, although there was a trend of UV-C having slightly lower seed weight at specific growth stages, which could be related to a higher respiration rate (Figure 35). Lower dry seed weight for UV-C treatment as compared to control was significant ($p<0.05$) at two days after treatment (Figure 34). Apart from a probable increase in respiration rate, this decrease in dry matter components, especially in UV-C treated seeds, could be related to inactivation of IAA by cationic peroxidases, which degrade IAA in the presence of oxygen (Chen and others 2002). It has been shown that cationic peroxidases (those participating in lignin formation) are activated due to UV-C stress (Murphy and Huerta 1990). Decreases in total mungbean biomass (27%) have previously been observed upon UV-B irradiation and conclusions on UV-B effect on mungbean growth and biomass were of a possible direct photooxidation effect on IAA (Pal and others 1999).

Soluble phenolics, cell wall-bound phenolics, lignin and antioxidant activity

Whole seeds

Figure 36 shows soluble phenolics being continuously synthesized throughout the germination period following zero order (synthesis/transformation) kinetics. The increase in soluble phenolics during the 112 h germination period was ~12-fold. Upon UV-C stress, mungbean seeds responded by synthesizing more soluble phenolics than controls. Significant differences (p-value <0.05) were observed starting at 24 h after UV-C treatment, where UV-C treated seeds had 17% higher soluble phenolics than controls (Figure 36). This lag time in phenolic synthesis is necessary for signal transduction processes to take place, including activation/synthesis of secondary messengers and phenylpropanoid enzymes such as PAL (Zhao and others 2005). Two days after UV-C treatment these differences increased (32% higher levels with UV-C, p-value <0.05) and three days after UV-C treatment, a 9% higher level was not significant (p-value >0.05); however significant differences (p-value <0.05) were observed with results expressed on seed basis (data not shown). This decrease in synthesis at the final sampling time could be related to oxidation of phenolics due to UV-C enhanced synthesis of acidic peroxidases, which oxidize phenolics (Ros Barcelo and others 2003). Work in abaxial leaf surfaces showed that UV-C induction of *trans*-resveratrol, a phenolic compound, peaks 20 h after irradiation, followed by a decrease due to possible oxidation or compound transformation (Ros Barcelo and others 2003).

Antioxidant activity of the synthesized phenolics in our study showed similar trends as for soluble phenolic content, an 8-fold increase due to germination and higher levels for UV-C treated seeds compared to controls but at different rates (Figure 37). Higher rates of phenolic antioxidant synthesis at initial growth stages and lower rates of phenolic antioxidant synthesis at later stages, indicate the presence of phenolics with higher antioxidant activity between water imbibition and one day after imbibition, and oxidation of phenolic antioxidants at later stages, thus reinforcing results previously presented in Chapters II and III. The rate of synthesis of antioxidant phenolic

compounds in response to UV-C stress also decreased at increasing growth times, thus showing transient increases in soluble phenolic antioxidants which are later transformed for functions other than antioxidants (Figure 37).

Results for cell wall bound phenolics are related to those hydroxycinnamic acid derivatives that are esterified to the cell wall and which are selectively released after alkaline hydrolysis (Ascensao and Dubery 2003). Due to their presence in the cell wall, they could be easily cross-linked with lignin for adding protection against biotic or abiotic stressors; or could exert independent barrier protective functions (Ascensao and Dubery 2003). Results showed a decreased content of cell wall bound phenolics in control seeds starting at 32 h after imbibition (Figure 38). This decrease could be due to cross-linking esterification of cell wall bound phenolics with lignin, thus strengthening and protecting cell walls against pathogens and environmental factors (Ascensao and Dubery 2003). Campbell and Ellis (1992) suggested that the occurrence of cell wall-esterified phenolics such as ferulic acid, could be related to lignin formation. Apart from cell wall bound phenolic decrease due to lignification, it is possible that bound phenolics were hydrolyzed and converted back into soluble phenolics due to cell wall loosening during this active growth period catalyzed by expansins, hydrolases and xyloglucan endotransglycosilase (Rodriguez and others 2002).

When UV-C stress was applied to mungbean seeds, there was a higher synthesis of cell wall bound phenolics as compared to controls, starting at 24 h after treatment (Figure 38). Higher levels (36% higher) of bound phenolics were observed at 24 h and 72 h after UV-C treatment when compared to controls at the same assayed times (Figure 38). These results complement others indicating that cell wall bound phenolics increase in response to biotic and abiotic stressors (Keller and others 1996, Ascensao and Dubery 2003). Increases in bound phenolics seem to be correlated with increases in soluble phenolics, indicating that cell wall bound phenolics could be derived from soluble phenolics via esterificaiton reactions. Some of the synthesized soluble phenolics could have cell functions such as cytoplasm antioxidants while others, such as *p*-coumaric, ferulic and sinapic acids, could be utilized as lignin precursors (Davin and Lewis 1992),

while most of the cell wall-bound phenolics are important as cell wall barrier protectors due to lignin strengthening (Ascensao and Dubery 2003). Differences in the type of phenylpropanoids synthesized are due to activation of different phenylpropanoid enzymes downstream of PAL (Douglas and others 1992).

For interpretation of lignin results, it is necessary to describe what lignin is. Lignin is a heteropolymer composed of *p*-hydroxyphenyl, guaiacyl and syringyl building blocks derived from the oxidative polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols (Ascensao and Dubery 2003). Simple phenolic precursors for these compounds include *p*-coumaric acid, ferulic acid and sinapic acid (Davin and Lewis 1992), while other hydroxycinnamic acid esters have been related to lignin cross-linking (Ascensao and Dubery 2003). Lignin has been separated into two classes, "soluble" and "core" lignin, with soluble lignins being lower molecular weight molecules that can be dissolved in mild alkali solution (Chen and others 2003). In our work, "core" lignin determinations were based on digestion of the saponified alcohol insoluble residue with 25% acetyl bromide in acetic acid, therefore excluding "soluble" lignin and lignin-like phenolic polymers from lignin quantifications (Ascensao and Dubery 2003).

Regarding initial lignin content, we observed that lignin levels decreased 51% (p-value <0.01) from dry seed to water imbibed seed (Figure 39). The higher lignin levels in dry mungbean seed could have a purpose of preventing herbivore feeding and digestion (Chen and others 2003). After seed digestion, animals will expel the intact seeds, thus allowing seeds to grow in diverse locations due to the help of animal dispersal. The sharp decrease in lignin content from dry to imbibed seed could be due to lignin breakdown and contribution to the plant carbon pool. It has been asserted that lignin constitutes 25% of plant global net primary production (NPP), being surpassed only by cellulose (Amthor 2003). Starting at imbibition stage, there was a constant increase in lignin content of control mungbean seeds throughout the tested germination period (36% increase from imbibed seed to final sampling, p-value <0.05) (Figure 39). When seeds were exposed to UV-C there was an increase in lignin synthesis, starting at 24 h after treatment (19% higher than 24 h control, p-value <0.05). Having similar lag

time as soluble and cell wall bound phenolics would indicate that enzymes related to lignin biosynthesis (i.e. cinnamoyl-CoA:NADP oxidoreductase, cinnamyl alcohol dehydrogenase) are activated at similar times as other enzymes of phenylpropanoid metabolism (i.e. PAL). Lignin synthesis in response to UV-C seems to be mediated by H_2O_2 and most likely is related to physical cell protection against this abiotic stressor (Ros Barcelo and others 2003). Three days after UV-C treatment, differences in lignin content of control and treatment were less evident, but still significant (6% higher than control, p-value <0.05). These decreases in lignin synthesis at later stages could indicate that the seed is well lignified and no further lignification is required to protect against the foreign environmental signals.

Increases in lignin are important from a plant physiological as well as human physiological standpoint, due to lignin's health benefit properties as dietary fiber (Harris and Ferguson 1999). Lignins are important due to possible anti-cancer properties, especially in relation to breast and colon cancer (Harris and Ferguson 1999). Lignins seem to flush excess estrogen from the body, thus reducing likelihood of estrogen-related cancers (i.e. breast cancer) (Arts and others 1991). Other properties attributed to lignins include antibacterial, antifungal, antiviral and antimutagenic (Sakagami and others 1991, Barber and others 2000, Krizkova and others 2000).

Seed sections

Measurement of soluble phenolics, cell wall-bound phenolics, lignin and TAC were also conducted on seed sections for determining the plant parts responsible for the enhanced phenylpropanoid metabolism and where compound mobilization/transformation takes place within sections. This information may be of nutritional and commercial significance since it is possible to identify concentrated sources of phenolic antioxidants for exploiting their nutraceutical properties.

Results in cotyledon sections indicate that soluble phenolics and antioxidant properties increased at a relatively high rate throughout the tested germination period, whereas bound phenolics and lignin also increased throughout germination but at lower

rates (Figure 40). These results indicate that the cotyledon seems to be a constant source of soluble phenolics for the growing seedling. This involvement of cotyledon in providing key precursors for a growing hypocotyl has also been proposed by Shetty and others (2001). Regarding the effect of UV-C stress on the cotyledon, results showed that phenylpropanoid enhancements were observed only for soluble phenolics (27% and 23% higher content than controls at 24 h and 72 h after treatment, respectively), but not for cell wall-bound phenolics or lignin (Figure 40). This indicates that UV-C stress on cotyledon tissue is activating enzymes related to synthesis of soluble phenolics, but not activating those related to cell wall-bound phenolics and lignins in this seed section. A 16% lower (p-value <0.05) level of cell wall bound phenolics and a 5% lower (p-value >0.05) lignin content at day 3 after UV-C exposure of treatments compared to controls could indicate a mobilization of signal transducers from cotyledon to the hypocotyl and roots section which require cell wall bound phenolics for physical protection via lignin cross-linking due to a rapid elongation stage (Passardi and others 2005).

Results for control hypocotyl section showed a slight decrease in the levels of soluble phenolics and antioxidant activity throughout germination (Figure 41). Regarding cell wall bound phenolics, this decrease was more dramatic (48% decrease from treatment application time to final sampling) and with lignin there was a 32% increase from treatment application time to final sampling. These results indicate that soluble phenolics seem to be transformed into cell wall-bound phenolics and immediately cross-linked with lignin (Ascensao and Dubery 2003). Results for UV-C treated seeds show transient increases in soluble phenolics, antioxidant properties, and cell wall bound phenolics due to enhancement of phenylpropanoid metabolism through probable PAL activation/synthesis. These transient increases indicate their immediate need for serving antioxidant functions and for lignifying hypocotyl and root tissues for protection functions. Increases in soluble phenolics, TAC and cell wall bound phenolics at 24 h after UV-C treatment, were 88%, 211% and 73%, respectively, when compared to controls at the same assayed time (Figure 41). Higher increases in antioxidant activity than in soluble phenolics will be related to increases in phenolics with a higher number

of antiradical-efficient hydroxyl groups. In general, decreases in soluble and bound phenolics at later germination stages of control and UV-C treated mungbean seeds could be due to their oxidation and incorporation into lignin. Some decreases in bound phenolics in control hypocotyl tissue with germination could be due to their hydrolysis into soluble phenolics due to increased cell wall loosening during active cell expansion catalyzed by hydrolases and other lytic enzymes (Rodriguez and others 2002).

Regarding the seed coat, we observed that throughout growth, the level of soluble phenolics and antioxidant properties decreased throughout germination, while there was an increase in bound phenolics and lignin (Figure 42). Antioxidant properties of soluble phenolics in the seed coat decreased by 77% between initial and final sampling. These results indicate that soluble phenolics could be oxidized and/or transformed into cell wall bound phenolics and lignin precursors. In relation to UV-C stress, there was no clear effect on the different parameters tested (Figure 42).

Regarding the level of soluble and bound phenolics and lignin contributed by the different sections, cotyledon section overall, due to a large contribution of dry matter per seed (55% to 93%), was the greatest contributor at the different growth stages assayed (Tables 4, 5). At imbibition stage, the greatest contributor of soluble phenolics and TAC was the seed coat, representing contributions of 53% and 67%, respectively (Table 5). Even though the seed coat represents only 7% of the total seed dry weight, it is a very concentrated source of phenolic antioxidants at imbibition stage and could be attractive for the nutraceutical market. At other stages, contributions of soluble phenolics and TAC for seed coat decreased with an increase in growth time, while those for cotyledon and hypocotyl increased due to increases in phenylpropanoid synthesis and or dry matter (Tables 4, 5). For cell wall bound phenolics and lignin, contributions were the highest for cotyledon section; however as germination progressed, % contributions decreased from 83% to 54%, and from 87% to 52%, for bound phenolics and lignin, respectively (Table 5). Increases in bound phenolics and lignin contributions of hypocotyl as germination progressed were observed, while contributions by seed coat remained relatively unchanged (Table 5). The high contributions of hypocotyl are related to

increases in hypocotyl and root dry matter, and in lignification and lignin precursors of these highly growth-active sections.

For UV-C treatments total contributions of soluble phenolics for cotyledon increased from 55% to 64% at day 2 after imbibition and from 47% to 59% at day 4 after imbibition, when compared to contributions for controls (Table 5). Increases in TAC and cell wall bound phenolic contributions for cotyledon were only observed for seeds sampled 2 days after imbibition. Contributions of soluble phenolics for hypocotyl decreased, while contributions of TAC increased, when compared to controls (Table 5). For cell wall bound phenolics contributed by hypocotyl, there was a decrease at day 2 after imbibition and an increase at day 4 after imbibition. Regarding lignin, there were very small increases with UV-C stress in the amounts contributed by cotyledon and hypocotyl sections sampled 4 d after imbibition. Very small increases in lignin contribution and decreases in soluble phenolics contributed by hypocotyl in response to UV-C stress are due to a smaller hypocotyl 2 and 4 days after imbibition (Tables 4, 5). As mentioned before, this decrease in mungbean hypocotyl growth has been observed by other researchers (Chen and others 2002) and has been attributed to inactivation of IAA due to increased amounts of peroxidase. Regarding the seed coat, its contributions to the different parameters tested in response to UV-C stress were lower than those of controls, indicating a dilution effect due to enhanced phenylpropanoid synthesis in other seed sections.

Superoxide radical

Superoxide radical ($O_2^{\cdot -}$) quantifications were conducted on intact tissues, therefore $O_2^{\cdot -}$ levels will be those found in apoplastic regions, since $O_2^{\cdot -}$ cannot cross biological membranes (Vranova and others 2002). These apoplastic $O_2^{\cdot -}$ levels would be mainly generated by plasma membrane bound NADPH-oxidase.

Superoxide production showed a constant increase throughout germination (Figure 43), which could be associated to the synthesis of soluble phenolics (Figure 36). In addition, superoxide radical production correlated with the increase in respiration rate (Figure 35). Another potential function of superoxide radical and ROS in general is

related to cell growth and expansion. Rodriguez and others (2002) showed that ROS generated by NADPH-oxidase are essential for elongation growth in the expanding zone of maize leaf blades. Hypocotyl, epicotyl and root development throughout germination in our study could be related to increases in $O_2^{\cdot -}$.

UV-C treated seeds showed transient decreases in $O_2^{\cdot -}$ immediately after treatment (< 8 h) and continuos increases starting at 8 h after treatment (Figure 43). $O_2^{\cdot -}$ contents of UV-C treated seeds were always lower than controls, which could be due to a fast transformation of $O_2^{\cdot -}$ into H_2O_2 by the enzyme superoxide dismutase, especially at closer times following UV-C application (Vranova and others 2002). Another possibility could be methodology related, on which the quantified product that relates to superoxide content, adrenochrome, seems to be inhibited by catalase (Adak and others 1998). We speculate that catalase activity could have increased due to UV-C elicitation. Murphy and Huerta (1990) on UV-C irradiated rose cell suspensions showed a 31% increase in catalase (p-value >0.05).

Hydrogen peroxide

Initial tests for H_2O_2 content were conducted without breaking the whole tissue; however no detection of H_2O_2 was observed. It is possible that in the slow process of permeation throughout plant tissues, H_2O_2 could have been transformed by enzymes such as peroxidase and catalase (Dat and others 2000). However, when intact seeds were left in the reagent solution for H_2O_2 detection we observed that hypocotyls and roots were stained blue (Figures 44), therefore quantifications were conducted on grinded tissues. Location of H_2O_2 is not cell section-specific, since H_2O_2 can diffuse throughout the cell (Vranova and others 2002). The fact than only hypocotylar and root sections were stained blue could be related to a known involvement of H_2O_2 in lignification and elongation processes (Murphy and Huerta 1990, Rodriguez and others 2002). Higher level of staining was observed in younger roots, which are tissues requiring higher lignification (Figure 44C).

Results show that hydrogen peroxide levels of controls increased until 2 days after imbibition, then decreased at the remaining tested germination periods (Figure 45). These decreases could be related to increases in H_2O_2 degrading enzymes such as peroxidase (Figure 46) and catalase (Dat and others 2000). UV-C stress enhanced a rapid induction of H_2O_2, which was observed as early as 70 min after treatment showing 21% higher levels (p-value<0.05) than controls. These increases occurred until 160 min post treatment, after which H_2O_2 levels decreased, as was observed for controls, but at later germination stages. Similar results have been previously observed on UV-C irradiated *Rosa damascene* cell suspension, with peak H_2O_2 levels occurring 60-90 min after the stress preceded by an efflux of cellular K^+ (Murphy and Huerta 1990, Ros Barcelo and others 2003). In tobacco, UV-C induced H_2O_2 production seems to be mediated by the activation of specific mitogen-activated protein kinases (MAPKs) (Ros Barcelo and others 2003).

Transient increases in H_2O_2 could be related to activation of phenylpropanoid metabolism and the higher H_2O_2 production in the initial period for UV-C treatments would indicate that enough signaling was exerted towards secondary metabolite synthesis. Lower contents of H_2O_2 as compared to controls at later germination stages, also observed visually with blue staining of hypocotyls (Figure 44A, B), could also be related to UV-C activation of H_2O_2 transforming enzymes such as peroxidase (Figure 46) and catalase (Murphy and Huerta 1990).

Decreases in $O_2^{\cdot -}$ and transient increases in H_2O_2 in response to UV-C stress situate H_2O_2 as preferential signal molecule mediating UV-C stress in dark germinated mungbean seedlings. In addition superoxide radicals are limited in mobility to the apoplastic region since they cannot cross biological membranes and in signaling functions due to a short half-life (2-4 us) (Vranova and others 2002). On the other hand, half-life for H_2O_2 is much longer (1 ms) than that of superoxide radical and can diffuse some distance from its site of production (Vranova and others 2002). Both radical species during local oxidative stress can diffuse through the apoplast and transport a warning signal to neighboring cells.

Ethylene and respiration rate

As was discussed earlier, increases in respiration rate (Figure 35) throughout germination and in response to UV-C stress could be related to increases in $O_2^{\cdot-}$ production (Figure 43) and synthesis of soluble phenolics (Figure 36). Several biological processes, including respiration rate have been shown to be accelerated by UV-C on minimally processed lettuce and cabbage, fresh tomato, and zucchini squash (El-Ghaouth and Wilson 1995, Maharaj and others 1999, Allende and Artes 2003a, Erkan and others 2001, Gomez-Lopez and others 2005). Increases in respiration rate could be related to damaged cells due to ionizing effects of UV-C (Allende and Artes 2003b).

Regarding ethylene, there seemed to be a gradual decrease in ethylene production (42% decrease from treatment application time to day 3 after treatment) as germination progressed and no significant differences (p-value >0.01) were observed for UV-C treatments compared to controls at the evaluated germination stages (Figure 47). These results indicate that ethylene synthesis is not affected by UV-C stress on mungbean seedlings, therefore eliminating ethylene as a possible secondary messenger upstream or downstream of ROS. Absence of ethylene induction due to UV-C was also observed in zucchini squash fruit, accompanied by an increase in respiration rate (Erkan and others 2001). The higher observed ethylene levels at initial stages could serve signaling functions during germination, other than phenolic synthesis, such as lateral cell expansion, root and root hair formation, among others (Taiz and Zieger 1998f). Lack of signal mediation toward phenolic synthesis was shown previously (Chapter IV) since exogenous ethylene applications did not enhance phenolic synthesis in mungbean seeds.

PAL

Results showed PAL activity to increase proportionally with germination (Figure 48). These increases in PAL are known to be related to increases in soluble phenolics (Figure 36) and could be related to increases in respiration rate (Figure 35), $O_2^{\cdot-}$

production (Figure 43) and H_2O_2 production (Figure 45). Induction of PAL activity due to UV-C stress was very fast, showing 105% higher activity than control as early as 160 min after treatment application (Figure 48). Higher PAL activities of UV-C treated versus controls were observed until day 2 after treatment, where activities were not significantly different (p-value >0.05). Three days after UV-C treatment, PAL activities of controls were 57% higher than those of treatments (Figure 48). Signaling of H_2O_2 for PAL activity due to UV-C stress is very likely due to sooner increases for H_2O_2 and similar evolution patterns for both parameters during germination (Figures 45, 48).

POX

Peroxidases have two possible catalytic cycles depending on the required function, peroxidative and hydroxylic (Passardi and others 2005). Peroxidative involves oxidation of substrates and transformation of H_2O_2 to H_2O, while hydroxylic the production of ROS (Passardi and others 2005). Apart from this, peroxidases are classified into two major classes based on plant function, those utilizing guaiacol as the electron donor in vitro and those that utilize reduced glutathione (GSH), Cyt c, pyridine nucleotides, and ascorbate as the electron donors (Prasad and others 1995). The first group, includes those participating in lignification, degradation of IAA, cross-linking of cell wall polymers, ethylene biosynthesis, pathogen defense, and wound healing; while the second group those involved with H_2O_2 scavenging functions in the cell. POX activity results in this study will be for POX utilizing guaiacol, therefore showing functions in relation to the mentioned first group of peroxidases.

Results showed that POX activity increased proportionally with germination on controls and UV-C treated seeds but at higher rates on the latter (Figure 46). Induction of POX due to UV-C was observed at 24 h after treatment, showing 28% higher (p-value <0.05) activities than controls. Increases in POX activity of UV-C treatments compared to controls, were higher as germination progressed (75% and 78% higher than controls at days 2 and 3 after treatment application time, respectively). Being POX induced (Figure 46) at a later time than PAL (Figure 48), rules out the possibility of PAL being mediated

by ROS produced by the hydroxylic cycle of peroxidases. Increases in peroxidase activity for normal plant cell development functions and in response to both biotic and abiotic factors have been previously proposed (Ros Barcelo and others 2003). UV-C induced H_2O_2 production in plant cells has previously been shown to be accompanied by an increase in peroxidase activity for lignification functions (Murphy and Huerta 1990). As mentioned before, the observed increases in POX activity could be related to utilization of H_2O_2 for cross-linking reactions during lignification (Ascensao and Dubery 2003), as well as to IAA inactivation (Chen and others 2002). Inactivating the plant growth hormone IAA, would explain the decreased fresh and dry weights of whole UV-C irradiated seeds and hypocotyl sections when compared to controls.

Soluble protein content

Seed protein levels remained relatively unchanged for controls during germination; however, higher contents were observed for UV-C treated seeds starting at 48 h after treatment (Figure 49). Since this protein induction occurred beyond 24 h after UV-C treatment, therefore beyond PAL activation, it is speculated that the function of the synthesized proteins be other than those related to synthesis of phenylpropanoids. Such proteins could be pathogenesis-related proteins including proteins with antimicrobial activity (Chamnongpol and others 1998), since induction of pathogen resistance has been reported in various plant systems exposed to UV irradiation (Lers and others 1998). Carbazole alkaloids are an example of a product of some of these pathogen-related proteins triggered by UV irradiation (Pacher and others 2001).

The effect of superoxide radical inhibition on phenolic synthesis

Due to observed increases in ROS, such as $O_2^{\cdot -}$ (Figure 43) and H_2O_2 (Figure 45) during dark germination and UV-C stress, we targeted ROS as potential signal molecules mediating phenolic synthesis. Among the several potential sources of ROS during the sress-induced response, NADPH-oxidase and/or NADH-coupled peroxidase mediated were chosen since other potential sources like peroxisomes and chloroplasts produce

ROS in response to light (Dat and others 2000). For determining the potential mediation on the synthesis of phenolic compounds of ROS during dark germination and UV-C stress, DPI was used as inhibitor of plasma membrane bound NADPH-oxidase and/or NADH-coupled peroxidase. Different parameters were measured at initial conditions and at growth stages were significant differences were previously observed between control and UV-C treatment (Figures 36, 45, 46, 48). These parameters were soluble phenolics (48 h after treatment), H_2O_2 (70 min after treatment), PAL (8 h after treatment), and POX (48 h after treatment).

Dark germination effect

Results show that all the tested parameters increased with germination as was expected (Figures 50 to 53); however increases in H_2O_2 content were not significant (p-value >0.05) (Figure 51). Lower increases in H_2O_2, compared to what was expected, could be due to a shift in induction of ROS due to addition of 1.5 h dip during inhibitor applications. Increases in final controls and UV-C treatments for seeds without DPI were standardized to 100% for determining the effect of inhibitors on the different parameters tested. Therefore any value between 0% and 100% would indicate that DPI reduced the potential increase in that specific parameter by that specific percentage value. In general, DPI inhibitors caused a decrease in all evaluated parameters, except for H_2O_2, and some of these decreases followed a dose-response relationship (Figures 50 to 53). Results showed that synthesis of soluble phenolics during germination was reduced by around 62% with DPI inhibitors (Figure 50). On seed basis, this reduction for 250 uM DPI was 92% (data not shown). Regarding increases in PAL activity, DPI inhibitor reduced these increases by up to 87%, being no significantly different from the initial control (p-value <0.05) (Figure 52). For POX parameter, 250 uM DPI inhibited the increases in POX activity by 69% (Figure 53). Figure 54 shows that fresh and dry weights of control mungbean seeds 2 d after treatment were significantly affected by increasing concentrations of DPI. DPI affected the potential increases in fresh weight during 2 d germination by decreasing it up to 50%. Regarding dry weight, even though

there was no increase during germination, DPI inhibitor decreased dry weights by 9% (p-value <0.05).

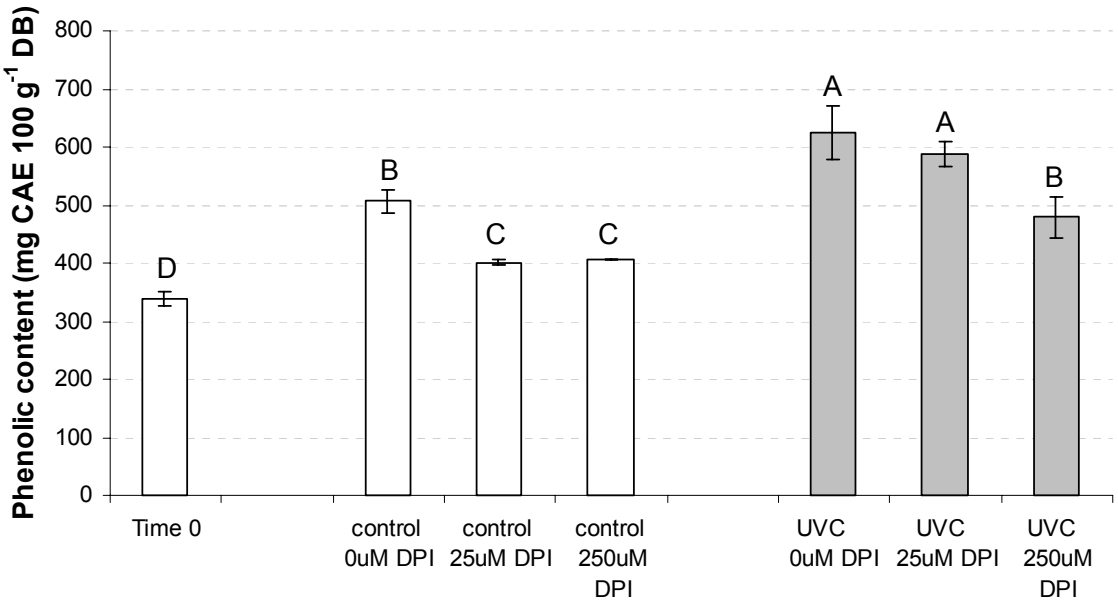

Figure 50 – Effect of ROS inhibition with DPI on soluble phenolic content of control and UV-C irradiated mungbean seeds evaluated 2 days after UV-C treatment. Similar letters for all samples indicate no significant differences (α = 0.05 with Duncan test) from each other. Data shows the average ± standard deviation, n = 4.

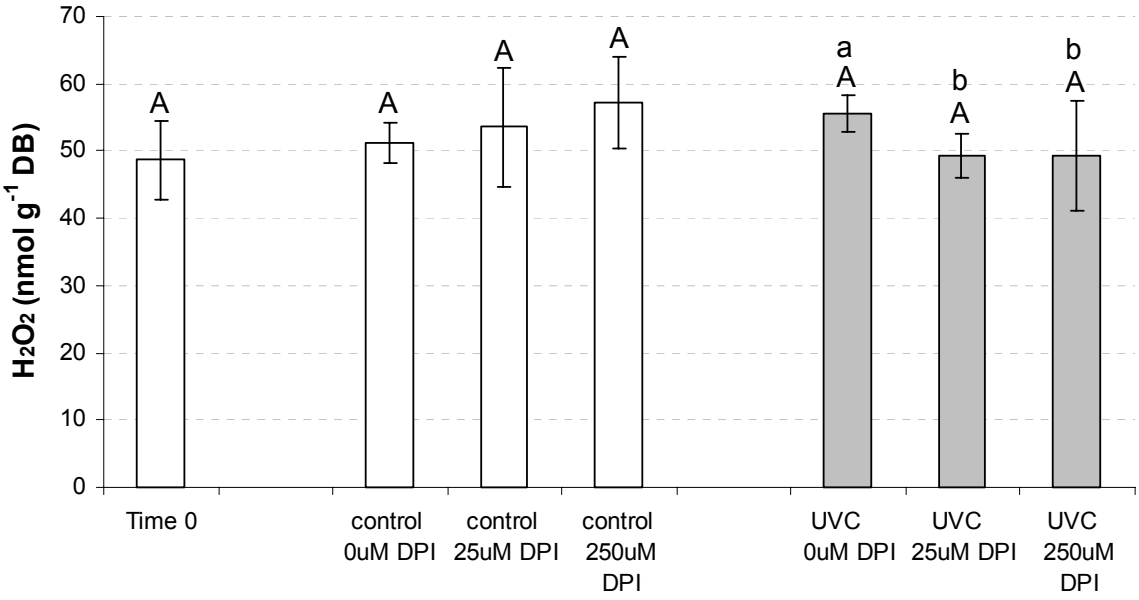

Figure 51 – Effect of ROS inhibition with DPI on hydrogen peroxide content of control and UV-C irradiated mungbean seeds evaluated 70 min after UV-C treatment. Similar upper case letters for all samples indicate no significant differences ($\alpha = 0.05$ with Duncan test) from each other. Similar lower case letters indicate no significant differences ($\alpha = 0.05$ with Duncan test) when UV-C treatments were compared to each other. Data shows the average ± standard deviation, n = 3.

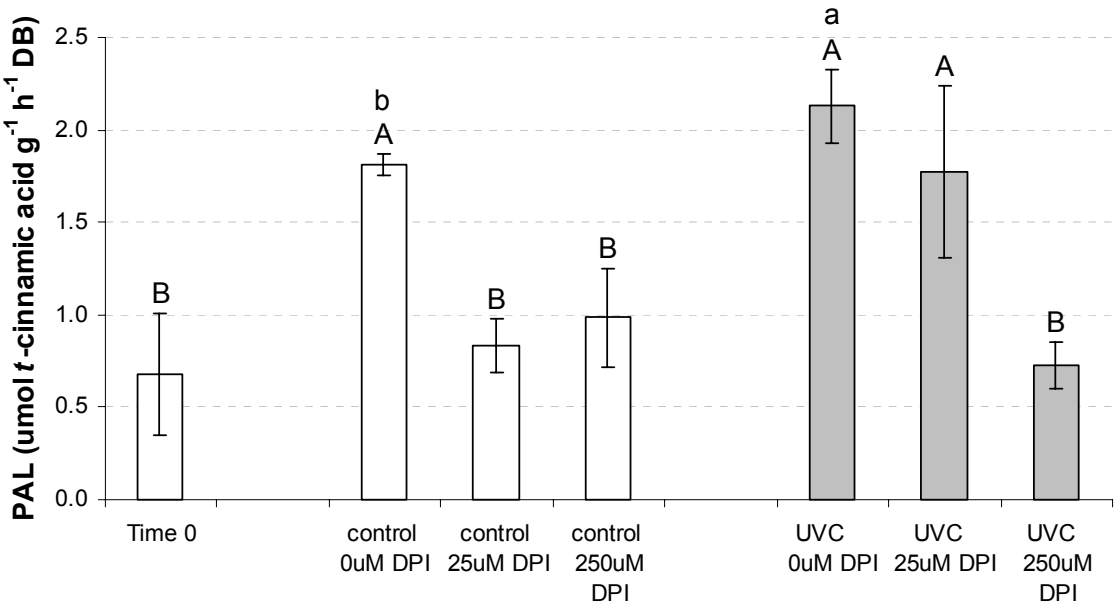

Figure 52 – Effect of ROS inhibition with DPI on phenylalanine ammonia lyase activity of control and UV-C irradiated mungbean seeds evaluated 8 h after UV-C treatment. Similar upper case letters for all samples indicate no significant differences ($\alpha = 0.05$ with Duncan test) from each other. Similar lower case letters indicate no significant differences ($\alpha = 0.05$ with Duncan test) when final UV-C was compared with final control, both without DPI. Data shows the average ± standard deviation, n = 4.

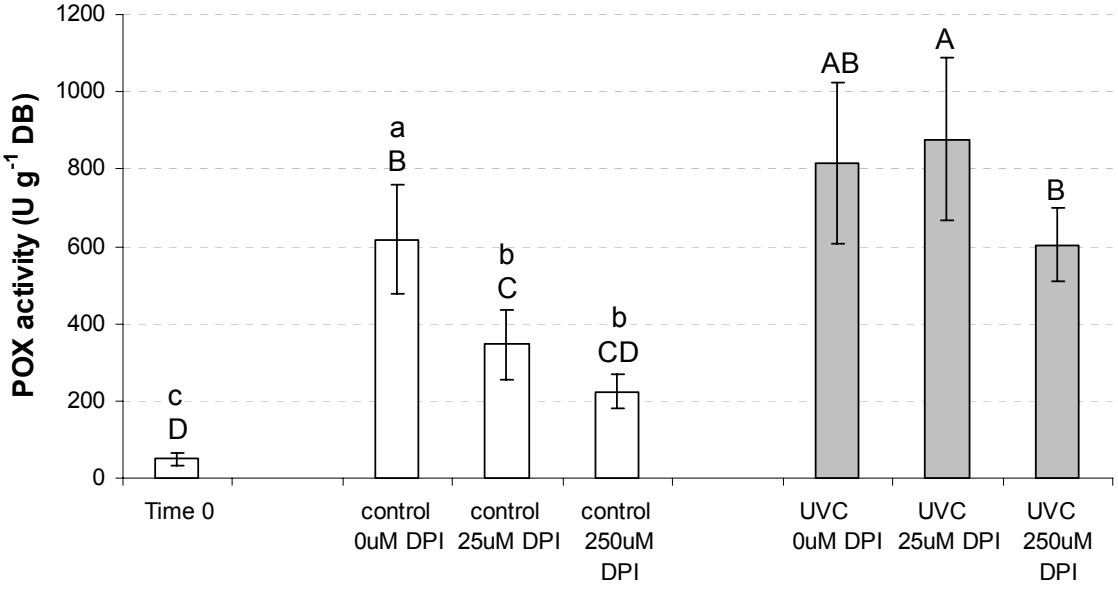

Figure 53 – Effect of ROS inhibition with DPI on guaiacol peroxidase activity of control and UV-C irradiated mungbean seeds evaluated 2 d after UV-C treatment. Similar upper case letters for all samples indicate no significant differences (α = 0.05 with Duncan test) from each other. Similar lower case letters indicate no significant differences (α = 0.05 with Duncan test) when non-UV-C treated seeds were compared to each other. Data shows the average ± standard deviation, n = 3.

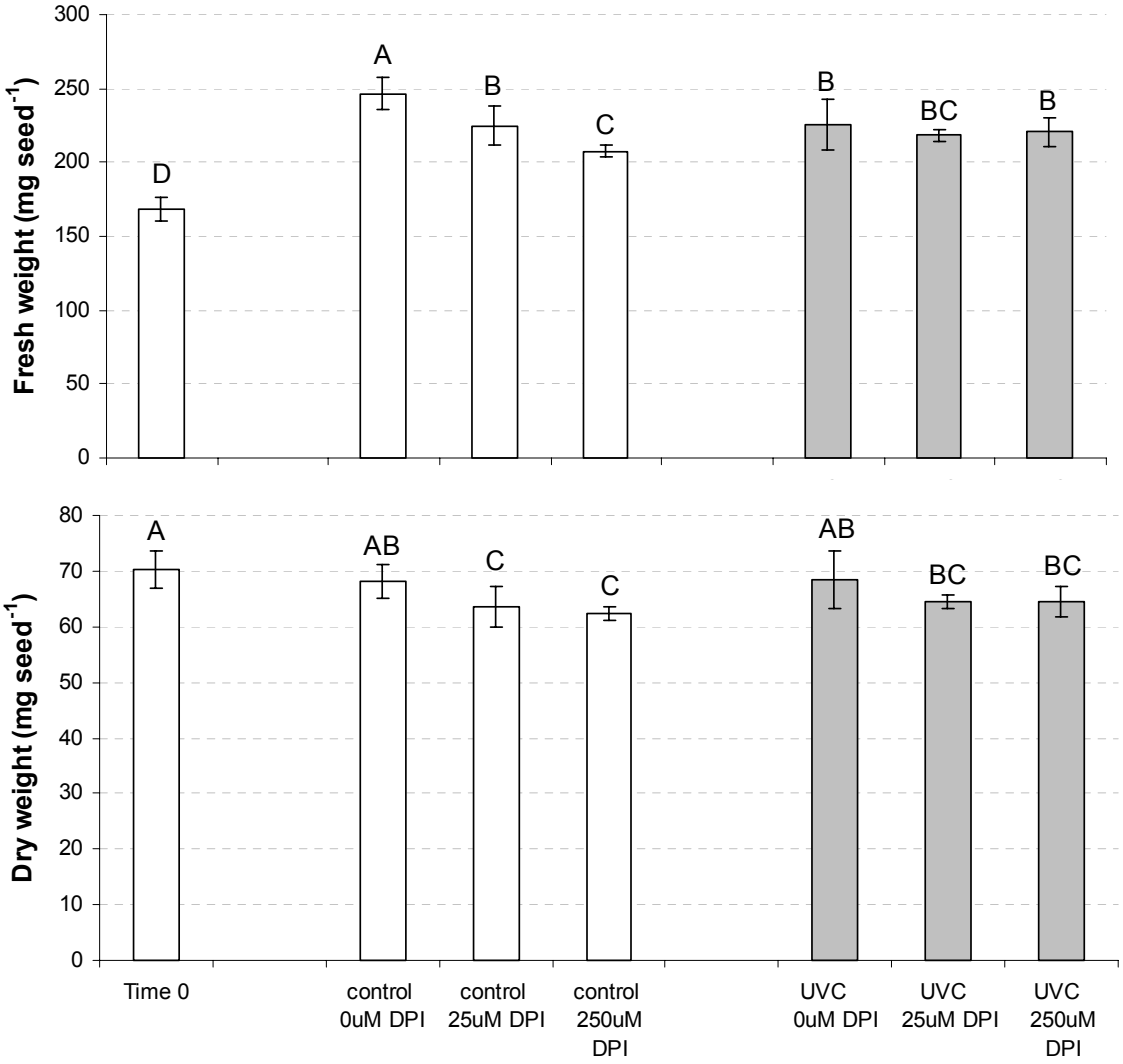

Figure 54 – Effect of ROS inhibition with DPI on fresh and dry seed weights of control and UV-C irradiated mungbean seeds evaluated 2 d after UV-C treatment. Similar letters for all samples within the same figure indicate no significant differences ($\alpha = 0.05$ with Duncan test) from each other. Data shows the average ± standard deviation, n = 6.

Overall, these results indicate that most, if not all the synthesis of soluble phenolics during germination are catalyzed by PAL and are signaled by ROS produced by membrane bound NADPH-oxidase. Regarding POX activity, these results show that POX is greatly affected by the presence or absence of superoxide radicals produced by NADPH-oxidase. It is possible that higher DPI levels could have further inhibited POX activity thus reaching full inhibition. Previous studies have shown that POX gene induction is triggered by H_2O_2 (Dat and others 2000), one of its preferred substrates. Due to a very fast dismutation of $O_2^{\cdot -}$ to H_2O_2 (Vranova and others 2002), having increased amounts of $O_2^{\cdot -}$ will yield higher amounts of H_2O_2, therefore higher POX activity. Results of POX inhibition are related to inhibition of signals affecting gene expression or activation of POX and not direct inhibition, since DPI does not directly inhibit the guaiacol oxidation activity (Frahry and Schopfer 1998). Regarding fresh and dry seed weights, these results showed that ROS produced from NADPH-oxidase are important for seedling growth and development. Rodriguez and others (2002) showed that ROS, presumably H_2O_2, are involved in growth expansion of embryonic axes, growing roots and germinating seeds. They specifically found that DPI significantly inhibited segment elongation on the expanding zone of maize leaf blades and the addition of H_2O_2 reverted this inhibition, indicating a clear role of ROS on growth and development of growing seedlings.

UV-C effect

UV-C yielded higher levels of all parameters tested than initial and final controls; however differences were not significant (p-value >0.05) from those of H_2O_2 controls (Figures 50 to 53). Dose response relationships on decreased level of the parameters tested with increasing DPI concentrations were observed for UV-C treated seeds (Figures 50 to 53). 250 uM DPI decreased the potential synthesis of soluble phenolics by 51% (Figure 50). For PAL, a decrease of 97% with 250 uM DPI and PAL activity values at 250 uM DPI not significantly different (p-value <0.05) from those of initial control indicate that almost all synthesis or activation of PAL during the tested period

was mediated by ROS from NADPH-oxidase (Figure 52). Regarding H_2O_2, also almost complete inhibitions were achieved with DPI (93%) (Figure 51). For POX, inhibitions were up to 28%, indicating that partial POX activation was signaled by ROS from NADPH-oxidase (Figure 53).

The effect of decreased fresh and dry seed weight due to DPI inhibitor observed for controls, was also observed for UV-C treated seeds; however no significant differences (p-value >0.05) were detected. Lower effects on UV-C treated seeds could be due to higher amounts of elicited ROS; therefore requiring higher DPI concentrations to achieve significant decreases in weight.

Similar inhibition effects on H_2O_2 and PAL indicate that inhibiting H_2O_2 production by inhibiting its precursor $O_2^{\cdot -}$, would inhibit H_2O_2 mediated PAL activation and/or synthesis. H_2O_2 or H_2O_2 downstream signals seem to be the only PAL messengers regulating response of mungbean seeds to germination and UV-C stress. Regarding the partial inhibition of soluble phenolics, it is possible that the small level of non-inhibited PAL (3%) would be responsible for synthesis of the 49% of phenolics that were not inhibited by the use of DPI. Another explanation could be that those soluble phenolics not inhibited with DPI could have being generated from hydrolysis of cell wall-bound phenolics to soluble phenolics due to direct UV-C effect or indirect effect due to apoplastic alkalinization potentially caused by known increases of K^+ efflux as a response to UV-C (Murphy and Huerta 1990). A lower inhibition of POX activity with DPI of UV-C treated as compared to controls, could be explained by POX activation signals other than H_2O_2, especially in response to UV-C stress. Ros Barcelo and others (2003) reviewed that a separate application of 14 different biotic and abiotic elicitors of H_2O_2 production was not always accompanied by an elicitation of peroxidase. It is also possible that ROS generated physically through the ionizing effects of UV-C could be participating in POX induction. Regarding lignin increases observed due to UV-C effect in Figure 39, we believe that they will decrease with DPI inhibitor, due to decreases in PAL mediated synthesis of lignin precursors as well as a shortage of H_2O_2 and decrease of peroxidases, essential elements for lignification (Passardi and others 2005).

Lack of involvement of guaiacol NADH-coupled peroxidases on the potential phenylpropanoid mediated synthesis by ROS induced by UV-C stress, was concluded earlier (Figures 46, 48). Together with results from this inhibition studies showing that almost all the UV-C elicited H_2O_2 and concurrent PAL activity are inhibited, we conclude that a great majority of phenolic synthesis due to UV-C is mediated by ROS produced by NADPH-oxidase and not by NADH-coupled peroxidases.

From the inhibition results with DPI, we propose that ROS are important for seedling growth and accumulation of biomass. Previous studies have presented ROS as promoters of elongation; however no mention about biomass has been done.

On Figure 55, we integrate a mechanistic diagram of phenylpropanoid induction due to dark germination and UV-C stress. Important components in this diagram include the role of ROS produced by NADPH oxidase on activation of phenylpropanoid synthesis and on seed growth regulation. Also shown is the fate of soluble and cell wall bound phenolics. The effect of increased POX activity on IAA inactivation and subsequent growth reduction are also depicted as well as possible effect of ROS on enhancing growth and biomass. Not depicted in this diagram include possible hydrolysis of cell wall bound phenolics due to UV-C stress either by direct action or by indirect action via apoplast alkalinization due to an increase in K^+ efflux.

Conclusions

From this chapter we determined phenolics to be important compounds during dark germination and important for protecting a growing seedling against UV-C stress. Signal transduction processes for UV-C stress started with transient increases in the levels of H_2O_2 together with increases in respiration, followed by increases in PAL activity, then synthesis of soluble/cell wall bound phenolics, followed by POX activity and lignin synthesis. From the different seed sections tested, cotyledon seemed to be the main source of phenylpropanoids and/or related signals for synthesis in hypocotyl and root sections. Hypocotyl and root sections, showed a high metabolic activity demanding

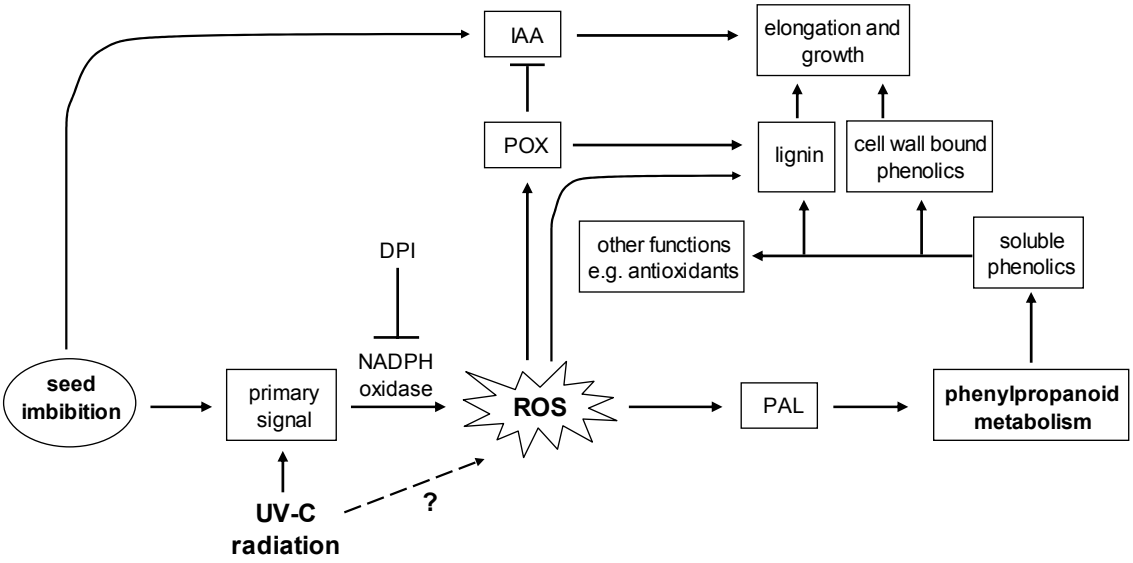

Figure 55 – Proposed mechanisms by which dark germination and UV-C irradiation induce and affect the synthesis and transformation of phenolic compounds in mungbean seedlings as well as the effects on peroxidase activity and seedling growth. DPI (diphenyleneiodonium chloride), ROS (reactive oxygen species), PAL (phenylalanine ammonia lyase), POX (guaiacol peroxidase), IAA (indole-3-acetic acid).

precursors for lignification due to active growth, which caused contributions to phenylpropanoid contents of whole seed to increase with germination.

Regarding ROS, we conclude that apart from known detrimental effects such as triggering DNA mutations, plant death and apoptosis, ROS can also protect the plant and promote growth throughout a normal germination process and upon exposure to abiotic stress such as high UV-C irradiation. We propose that the main synthesis of soluble phenolics during dark germination and UV-C stress is mediated by ROS synthesized by plasma membrane bound NADPH dependent oxidase and not by ROS from other sources such as peroxidases or light dependent organelles. With UV-C stress, enzyme mediation by ROS produced physically due to high energy ionizing effects is possible and could complement mediation by ROS from NADPH oxidase; however, further experiments are needed to confirm this.

From information generated in this Chapter and Chapter IV, we speculate that H_2O_2 is the mediator signal rather than $O_2^{\cdot-}$; however, further tests should confirm this hypothesis. We rule out the possibility of phenylpropanoid mediated synthesis by ethylene downstream of ROS due to UV-C stress; however leave open probable secondary mediators downstream of ROS such as jasmonic acid (Conconi and others 1996). Due to a probable role of H_2O_2 as activator of phenylpropanoid metabolism it will be interesting to test if the level of phenolic compounds with antioxidant activity increase in the presence of a catalase inhibitor such as 3-aminotriazole or methyl viologen.

Potential health benefit properties of mungbean seeds were increased by dark germination and further enhanced by UV-C stress, thus showing potential tools which could benefit Food and Nutraceutical Industries by enhancing health benefits and adding value to their fresh or processed products.

CHAPTER VI

GENERAL CONCLUSIONS AND RECOMMENDATIONS

In this study, seedlings were determined to be small-scale phenolic producing factories and we propose their exploitation as such. The amount and type of phenolics synthesized by these seedlings will depend on the conditions on which they grow. By understanding how seeds respond to normal and adverse growing conditions it is possible to control the desired profile (quality and amount) of synthesized phenolic compounds. Increasing the synthesis of these health-giving compounds would add-value to plant-derived food products for applications in fresh and processed food markets. By implementing combination strategies of the different factors studied (i.e. germination time, temperature, wounding, UV-C) it is possible to optimize the synthesis of phenolic compounds with the desired health benefit or other properties.

We observed that the most important roles of synthesized phenolics in mungbean seedlings during germination, wounding and UV-C seem to be for lignification and for serving as phytoalexins. Even though lignin has health benefits due to its role as dietary fiber, synthesis of soluble phenolics could be further enhanced in follow-up studies by preventing their partial transformation into lignin. This could be achieved by partially inhibiting or controlling enzymes related to lignification (i.e. peroxidase). Outcomes of this inhibition could be reduced seedling growth and weakening of the mechanical structure; however, it could yield a "functional sprout" with higher nutraceutical concentrations.

Regarding hydrogen peroxide, we propose this molecule to be multi functional during germination. We determined ROS to be essential for phenolic synthesis and to play a key role as a growth regulator; however, further experiments are needed to confirm the latter. Other unresolved issues for further investigation include the causes and effects of a potential triggering of the oxidative burst during water imbibition and its association to dormancy rupture. In regards to guaiacol peroxidase, it will be interesting to determine the exact mechanism by which its activity or synthesis increases due to

UV-C stress. We determined some induction of peroxidase to be mediated by NADPH oxidase; however, we also propose the involvement of ROS generated physically by UV-C through water ionization, in addition to those generated enzymatically.

For ensuring overall enhanced health benefits of plant tissues through our proposed approach, we recommend follow-up studies on the evaluation of other health-giving and promoting compounds such as proteins, fatty acids, vitamins, minerals, carotenoids and other phytochemicals; complemented with toxicity studies. Once this is accomplished, then we could rest assured knowing that a "functional sprout" would be healthier for consumers.

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VITA

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